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(71) Applicant *(for all designated States except US)*: MOUNT SINAI HOSPITAL CORPORATION [CA/CA]; 600 University Avenue, Toronto, Ontario M5G 1X5 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PAWSON, Anthony [CA/CA]; 34 Glenwood Avenue, Toronto, Ontario M6P 3C6 (CA). HENKEMEYER, Mark [US/CA]; Apartment 503, 263 Adelaide Street West, Toronto, Ontario M5T 1K5 (CA). LETWIN, Kenneth [CA/CA]; Apartment 1010, 20 Carluke Crescent, Willowdale, Ontario M2L 2J1 (CA).

(74) Agent: BERESKIN & PARR; 40th floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).

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(57) Abstract

Novel receptor tyrosine kinase protein and isoforms thereof which are expressed in migrating axons, and nucleic acid molecules encoding the novel protein isoforms and parts thereof are disclosed. The invention also relates to methods for identifying substances which are capable of binding to the receptor protein and methods for screening for agonists or antagonists of the binding of the protein and substance. Diagnostic and therapeutic methods using the protein and nucleic acid molecules are also described.

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Neural receptor tyrosine kinase

FIELD OF THE INVENTION

The invention relates to a novel receptor tyrosine kinase protein and isoforms and parts thereof, nucleic acid molecules encoding the novel protein and fragments thereof, and uses of the protein and nucleic acid molecules.

BACKGROUND OF THE INVENTION

Embryonic development of multicellular organisms is a highly ordered process that requires coordination of individual cells. Every cell must decipher the numerous signals it receives and then properly execute commands in order to achieve the correct position and differentiated state in the animal. The exquisite controls over cell growth, determination, migration and adhesion are mediated by molecules located on the plasma membrane surface.

A class of membrane associated molecules known to regulate cellular interactions are receptor tyrosine kinase proteins. The evolutionary conservation of genes encoding receptor tyrosine kinase proteins and their targets has emphasized the importance of these proteins in intracellular communication, and has also provided model systems for genetic analysis of tyrosine kinase signalling pathways. Such studies have shown that some tyrosine kinases function to specify a particular cell fate, such as the sevenless (sev) receptor in Drosophila R7 photoreceptor cells and the Let-23 receptor in nematode vulval cells (reviewed by Greenwald and Rubin, *Cell* 68:271-281, 1992). The binding of sev with its ligand, boss, results in cell clustering suggesting a role in cell-cell adhesion for these molecules (Kramer *et al.*, *Nature* 352:207-212, 1991). The receptor tyrosine kinase encoded by *torso* functions in pattern formation by specifying the terminal poles of Drosophila embryos (Sprenger *et al.*, *Nature* 338:478-483, 1989). Genetic analysis has recently provided insight into the functions of a small number of receptor tyrosine kinases in mouse development, including the α-platelet-derived growth factor receptor, the colony stimulating factor-1 receptor, and c-Kit/*W* (Pawson and Bernstein, *Trends in Genetics* 6:350-356, 1990).

A growing number of closely related transmembrane receptor tyrosine kinase proteins containing cell adhesion-like domains on their extracelluar surface have recently been identified. Collectively, this group of proteins defines the *Eph/Elk/Eck* subfamily, which is made up of at least fifteen related but unique gene sequences in higher vertebrates (Hirai *et al., Science* 238:1717-1720, 1987; Letwin *et al., Oncogene* 3:621-627, 1988; Lindberg *et al., Mol. Cell. Biol.* 10:6316-6324, 1990; Lhotak *et al., Mol. Cell. Biol.* 11:2496-2502, 1991; Chan and Watt, *Oncogene* 6:1057-1061, 1991; Lai and Lemke, *Neuron* 6:691-704, 1991; Pasquale, *Cell Regulation* 2:523-534, 1991; Sajjadi *et al., New Biologist* 3:769-778, 1991; Wicks *et al., PNAS* 89:1611-1615, 1992; Gilardi-Hebenstreit *et al., Oncogene* 7:2499-2506, 1992; Bohme *et al., Oncogene* 8:2857-2862, 1993; Sajjadi and Pasquale, *Oncogene* 8:1807-1813, 1993). *Eph* family members encode a structurally related cysteine rich extracelluar domain containing a single immunoglobulin (Ig)-like loop near the N-terminus and two fibronectin III (FN III) repeats adjacent to the plasma membrane. Examples of Eph family members include Cek5 (Pasquale, *Cell Regulation* 2:523-534, 1991) and *Erk*; (Chan and Watt, *Oncogene* 6:1057-1061 1991). Another *Eph* family member, *Sek*, has been shown to be segmentally expressed in specific rhombomeres of the mouse hindbrain (Nieto *et al., Development* 116:1137-1150, 1992). The presence of cell adhesion-like domains in this family of tyrosine kinases suggests that these proteins function in cell-cell interactions.

The other major families of proteins implicated in cell adhesion include the cadherins, selectins, integrins, and those of the immunoglobulin superfamily (reviewed by Hynes, R.O. and Landers, A.D., Cell 68, 303-322, 1992). The extracelluar regions of cell adhesion molecules frequently contain peptide repeats, such as FN III motifs, epidermal growth factor (EGF) repeats, or Ig loops that may direct protein-protein interactions at the cell surface. A number of cell adhesion molecules in both vertebrates (Dodd, J. and Jessell, T.M., Science, 242, 692-699, 1988; Jessell, T.M., Neuron, 1, 3-13, 1988; Furley *et al.*, Cell 61, 157-170, 1990; Burns *et al.*, Neuron, 7, 209-220, 1991) and invertebrates (Bastiani et al., *Cell* 48:745-755, 1987; Elkins *et al., Cell* 60:565-575,

1990; Grenningloh *et al., Cold Spring Harb. Symp. Quant. Biol.* 55, 327-340, 1991; Nose *et al., Cell* 70:553-567, 1992) have been implicated in axonal growth cone guidance and pathway/target recognition. Other aspects of neuronal morphogenesis involving cell-cell interactions may also require the activities of cell adhesion molecules (Edelman and Thiery, In *The Cell in Contact: Adhesions and Junctions as Morphogenetic Determinants*, Wiley, New York, 1985; Hatta *et al., Dev. Biol.* 120:215-227, 1987; Takeichi, *Development* 102:639-655, 1988; Takeichi, *Annu. Rev. Biochem.* 59:237-252 1990; Takeichi, *Science* 251:1451-1455, 1991; Edelman, *Biochemistry* 27:3533-3543, 1988; Grumet, *Curr. Opin. Neurobiol.* 1:370-376, 1991; Hynes and Lander, *Cell* 68:303-322, 1992). For example, ectopic N-cadherin expression during gastrulation stage Xenopus embryos has been shown to interfere with segregation of the neural tube from the ectoderm (Detrick *et al., Neuron* 4:493-506, 1990; Fujimori *et al., Development* 110:97-104, 1990). Although many different types of cell adhesion molecules have been identified, little is known about how these adhesive interactions are regulated and how they function in cell signalling pathways during normal development.

A critical stage in the development of the nervous system is the projection of axons to their targets. Navigational decisions are made at the growth cones of the migrating axons. As axons grow their growth cones extend and retract filopodia and lamellipodia processes which are implicated in the navigational decisions and pathfinding abilities of migrating axons. Like peripheral nervous system axons, the growth cones of neurons associated with the central nervous system follow stereotyped pathways and apparently can selectively chose from a number of possible routes (reviewed by Goodman and Shatz, *Cell* 72:77-98, 1993). Early pathways in the vertebrate embryonic brain are thought to be arranged as a set of longitudinal tracts connected by commissures. However, the molecular mechanisms that underly growth cone navigation and axon pathfinding in development are poorly understood (Hynes, R.O. and Lander, A.D., 1992, Cell 68:303).

Evidence indicates that the development of the endolymphatic duct is under the control of neuronal induction (Van De Water and Represa, Van

De Water, T.R. and Represa, J. (1991). *Ann. NY Acad.* 630:116-128, 1991). The endolymphatic duct pinches off from the otic vesicle and elongates to form a tube that apparently functions in regulating the endolymph fluid pressure in the membranous labyrinth of the internal ear (Guild, *Amer. J. Anat.* 39:57-81, 1927; Rugh, *The Mouse: Its reproduction and Development*. Minneapolis: Burgess, 1968; Sher, 1971; Hendriks and Toerien, 1973; Theiler, 1989; Kaufman, In *Postimplantation Mammalian Embryos: a Practical Approach* (ed. A.J. Copp and D.L. Cockroft) pp. 81-91. New York: Oxford University Press, 1990).

The developmental function of tyrosine kinases during axonogenesis has been studied in Drosophila. A function in axonal pathfinding is evident for the Drosophila abl tyrosine kinase when *abl* mutations are combined with mutations in other genes including the neural cell adhesion molecule, *fasciclin* I (*fas* I, Elkins *et al.*, *Cell* 60:565-575, 1990) or *disabled* (*dab*, Gertler *et al.*, *Cell* 58:103-113, 1989). These studies have shown that the abl tyrosine kinase is specifically localized to the axonal compartment of the embryonic Central Nervous System (CNS) (Gertler *et al.*, *Cell* 58:103-113, 1989). Moreover, genetic analysis has indicated that subcellular localization to axons is essential for abl function during development (Henkemeyer *et al.*, *Cell* 63:949-960, 1990) and that mutations in second-site modifier genes including *fas* I and *dab* can reveal a role for abl in axonogenesis (Elkins *et al.*, *Cell* 60:565-575, 1990; Gertler *et al.*, *Cell* 58:103-113 1989). The requirement for tyrosine phosphorylation in axonal outgrowth and adhesion in Drosophila is strengthened by the identification in CNS axons of three transmembrane tyrosine phosphatases containing FN III motifs (Tian *et al.*, *Cell* 67:675-685, 1991; Yang *et al.*, *Cell* 67:661-673, 1991).

SUMMARY OF THE INVENTION

The present inventors have identified and characterized a receptor tyrosine kinase protein that plays an important role in cell-cell interactions and axonogenesis in the development of the nervous system. In particular, the present inventors have cloned a novel murine gene, designated neural kinase (*Nuk*). The gene encodes a new member of the Eph subfamily of receptor tyrosine kinases, designated Nuk protein. The murine *Nuk* locus was

mapped to the distal end of mouse chromosome 4 near the *ahd*-1 mutation.

The biological function of Nuk protein was investigated using antibodies having anti-Nuk protein specificity. A detailed immunohistochemical analysis of its subcellular localization in whole-mount mouse embryos indicated that during early embryogenesis Nuk protein is confined to the developing nervous system, where it marks segments along the axis of the neural tube in the hindbrain (rhombomeres r2, r3, and r5) and specific morphological bulges of the midbrain and forebrain.

Nuk protein was also found to be concentrated at sites of cell-cell contact, often involving migrating neuronal cells or their extensions. Most notably, high levels of Nuk protein were found within initial axon outgrowths and associated nerve fibers, including most if not all peripheral nervous system (PNS) axons. The axonal localization of Nuk protein was also found to be transient and was not detected after migrations have ceased.

The subcellular localization of Nuk protein, as well as the presence of fibronectin type III and immunoglobulin-like adhesive domains on the extracelluar region, indicates that this receptor tyrosine kinase functions to regulate specific cell-cell interactions during early development of the nervous system and that it has a role during the early pathfinding and/or fasciculation stages of axonogenesis in animals.

The present invention therefore provides a purified and isolated nucleic acid molecule containing a sequence encoding a receptor tyrosine kinase protein which is expressed in migrating axons, or an oligonucleotide fragment of the sequence which is unique to the receptor tyrosine kinase protein. In a preferred embodiment of the invention, the purified and isolated nucleic acid molecule comprises (a) a nucleic acid sequence encoding a protein having the amino acid sequence as shown in SEQ ID NO:2 and Figure 2; (b) nucleic acid sequences complementary to (a); (c) nucleic acid sequences which are at least 97% identical to (a); or, (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.

Most preferably, the purified and isolated nucleic acid molecule

comprises (a) a nucleic acid sequence as shown in SEQ ID NO:1 and Figure 1, wherein T can also be U; (b) nucleic acid sequences complementary to (a); (c) nucleic acid sequences which are at least 85% identical to (a); or, (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.

It is contemplated that a nucleic acid molecule of the invention may be prepared having deletion and insertion mutations. For example, the extracelluar domain or parts thereof, such as the FN III and Ig domains; the transmembrane region or parts thereof; the tyrosine kinase domain or parts thereof, such as the ATP binding site and; the carboxy terminal tail may be deleted. In a preferred embodiment, the deletions are in a portion of the nucleic acid molecule of the invention encoding the extracelluar domain of Nuk protein, most preferably the portion comprising codons 29 to 50 in SEQ ID NO:1. In another preferred embodiment, the deletions are in a portion of the nucleic acid sequence of the invention encoding the kinase domain of Nuk protein, most preferably the portion comprising the ATP-binding site amino acid number 623-707.

The invention further contemplates a purified and isolated double stranded nucleic acid molecule containing a nucleic acid molecule of the invention or a fragment thereof, hydrogen bonded to a complementary nucleic acid base sequence.

The nucleic acid molecules of the invention, or fragments thereof may be inserted into an appropriate expression vector, i.e. a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Accordingly, recombinant DNA molecules adapted for transformation of a host cell may be constructed which comprise a nucleic acid molecule of the invention and one or more transcription and translation elements operatively linked to the nucleic acid molecule.

In an embodiment of the invention, a recombinant molecule is provided which contains a nucleic acid molecule of the invention having a deletion or insertion mutation. Such a recombinant molecule may further comprise a reporter gene.

The recombinant molecule can be used to prepare transformed host cells expressing the protein or part thereof encoded by a nucleic acid molecule of the invention. Therefore, the invention further provides host cells containing a recombinant molecule of the invention. The invention also contemplates transgenic non-human mammals whose germ cells and somatic cells contain a recombinant molecule of the invention.

The invention further provides a method for preparing a novel receptor tyrosine kinase protein or isoforms or parts thereof utilizing the purified and isolated nucleic acid molecules of the invention.

The invention further broadly contemplates a purified and isolated receptor tyrosine kinase protein which is expressed in migrating axons, or an isoform or a part of the protein. In a preferred embodiment, a purified receptor tyrosine kinase protein is provided which has the amino acid sequence as shown in SEQ ID NO:2 or Figure 2, or a sequence having between 97 and 100 percent identity thereto. The receptor tyrosine kinase protein of the invention may also be phosphorylated.

Conjugates of Nuk protein of the invention, or parts thereof, with other molecules, such as proteins or polypeptides, may be prepared. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins. In a preferred embodiment a fusion protein is provided comprising a part of the protein of the invention, preferably the extracelluar domain, most preferably having the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 26 to 548 or amino acids 600 to 618; or the carboxy terminal, most preferably having the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 601 to 994; or sequences having at least 97% identity thereto.

The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and accordingly to the novel receptor tyrosine kinase protein of the invention or a part of the protein. Thus, the invention also relates to a probe comprising a nucleotide sequence coding for a protein which displays the properties of the novel receptor tyrosine kinase of the invention or a part which is unique to

the protein. The probe may be labelled, for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleotide sequence coding for a protein which displays the properties of the novel receptor tyrosine kinase protein of the invention, or a part thereof.

The invention still further provides a method for identifying a substance which is capable of binding to the novel receptor tyrosine kinase protein of the invention, or an isoform or part of the protein, comprising reacting the novel receptor tyrosine kinase protein of the invention, or part of the protein, with at least one substance which potentially can bind with the receptor tyrosine kinase protein, isoform or part of the protein, under conditions which permit the formation of substance-receptor kinase protein complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for activation of the receptor tyrosine kinase proteins.

In an embodiment of the method, ligands are identified which are capable of binding to and activating the novel receptor tyrosine kinase protein of the invention. The extracellular ligands which bind to and activate the novel receptor tyrosine kinase protein of the invention may be identified by assaying for protein tyrosine kinase activity.

Still further, the invention provides a method for assaying a medium for the presence of an agonist or antagonist of the interaction of a receptor tyrosine kinase protein of the invention and a substance which binds to the receptor tyrosine kinase protein, preferably a ligand. In an embodiment, the method comprises providing a known concentration of a receptor tyrosine kinase protein of the invention, isoforms thereof, or part of the protein, preferably the extracelluar domain of the protein, incubating the receptor tyrosine kinase protein with a substance which is capable of binding to the receptor tyrosine kinase protein, isoforms thereof, or part of the protein, and a suspected agonist or antagonist substance under conditions which permit the formation of substance-receptor protein complexes, and assaying for substance-receptor protein complexes, for free substance, for non-complexed proteins, or for activation of the receptor tyrosine kinase protein. The methods

of the invention permit the identification of potential stimulators or inhibitors of axonal migration and nerve cell interactions in development and regeneration, which will be useful in the treatment of nerve disorders and nerve damage.

The invention also contemplates a method for identifying a substance which is capable of binding to an activated receptor tyrosine kinase protein of the invention, or an isoform or part of the activated protein, comprising reacting the activated receptor tyrosine kinase protein of the invention, or part of the protein, with at least one substance which potentially can bind with the receptor tyrosine kinase protein, isoform or part of the protein, under conditions which permit the formation of substance-receptor kinase protein complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for phosphorylation of the substance. In an embodiment of the method, intracellular ligands such as Src homology region 2 (SH2)-containing proteins which are capable of binding to a phosphorylated receptor tyrosine kinase protein of the invention, or intracellullar ligands which may be phosphorylated by the novel receptor tyrosine kinase of the invention may be identified.

The invention further contemplates antibodies having specificity against an epitope of the receptor tyrosine kinase protein of the invention or part of the protein which is unique to the receptor tyrosine kinase protein. Antibodies may be labelled with a detectable substance and they may be used to detect the novel receptor tyrosine kinase of the invention in tissues and cells. The antibodies may accordingly be used to monitor axonal migration and nerve cell interactions.

Substances which affect axonal migration may be identified using the methods of the invention by comparing the pattern and level of expression of the novel receptor tyrosine kinase protein of the invention in tissues and cells in the presence and in the absence of the substance. Thus the invention provides a method for screening for substances having pharmaceutical utility in the treatment and diagnosis of nerve disorders and nerve damage.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1 shows the nucleotide sequence encoding the novel receptor tyrosine kinase protein of the invention as shown in SEQ ID NO: 1;

Figure 2 shows the amino acid sequence of the novel receptor tyrosine kinase protein of the invention as shown in SEQ ID NO:2 and a schematic diagram of the regions of the novel receptor tyrosine kinase protein of the invention;

Figure 3A shows immunoprecipitation of the novel receptor tyrosine kinase protein of the invention;

Figure 3B shows Western Blot analysis of immunoprecipitates of the novel receptor tyrosine kinase protein of the invention;

Figure 4A shows localization of mRNA of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 7.5 day old mouse embryo;

Figure 4B shows localization of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 8 day old mouse embryo;

Figure 4C shows localization of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 8.75 day old mouse embryo;

Figure 4D shows localization of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 9.5 day old mouse embryo;

Figure 4E shows localization of the novel receptor tyrosine kinase

protein of the invention in whole-mount sections of 9.5 day old mouse embryo in greater detail than in Figure 4D;

Figure 4F shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin serial transverse sections of 9.5 day old mouse embryo;

Figure 4G shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin serial transverse sections of 9.5 day old mouse embryo;

Figure 4H shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin sagittal section along the midline of 10.5 day old mouse embryo;

Figure 4I shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin adjacent transverse sections of 10.5 day old mouse embryo immunoreacted with anti-Nuk protein antibodies;

Figure 4J shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin adjacent transverse sections of 10.5 day old mouse embryo immunoreacted with a trpE-Nuk peptide;

Figure 5A shows an adjacent transverse section of an 11.5 day embryo at the level of the caudal/posterior spinal cord immunoreacted with anti-Nuk antibodies;

Figures 5B shows an adjacent transverse section of an 11 day embryo at the level of the caudal/posterior spinal cord immunoreacted with anti-Nuk antibodies preincubated with a trpE-Nuk peptide;

Figures 5C shows transmission electron microscopy immunolocalization of Nuk protein in ventral midbrain cells of a 9.5 day embryo;

Figure 5D shows transmission electron microscopy (EM) immunolocalization of Nuk protein in ventral midbrain cells of a 9.5 day embryo;

Figure 6A is a photomicrograph of the head of an 11.5 day anti-Nuk whole-mount immunostained embryo with one of the pair of oculomotor nerve fibers in focus showing strong labelling for Nuk protein (filled arrow);

Figure 6B shows a clearer view of Nuk protein staining in the oculomotor axons fibers obtained when the whole-mount staged embryo was filleted down the midline to minimize tissue thickness;

Figure 6C shows a paraffin section of a 10.5 to 11 day mouse embryo immunostained with anti-Nuk;

Figure 6D shows a paraffin section of a 10.5 to 11 day mouse embryo immunostained with anti-Neurofilament antibodies.

Figure 6E is a frontal section immunostained with anti-Nuk antibodies which label the ventral midbrain and oculomotor axon fibers as they exit the neural tube (filled arrow) and extend (open arrow) towards their target tissue, the pre-optic muscle mass;

Figure 6F shows a sagittal section immunostained with anti-Nuk antibodies which label the oculomoter axon fascicule as it enters the pre-optic muscle mass (open arrow);

Figure 7A is a 10.5 day whole-mount embryo immunostained with anti-Nuk antibodies which label the trigeminal nerve V and facial nerve VII;

Figures 7B shows transverse sections of a 10.5 day embryo showing Nuk-positive trigeminal nerve V axon fascicules labelled with the anti-Nuk antibodies;

Figures 7C shows transverse sections of a 10.5 day embryo showing Nuk-positive trigeminal nerve V axon fascicules labelled with the anti-Nuk antibodies;

Figures 7D is an 11.5 day anti-Nuk whole-mount showing localization of Nuk protein in the vagus nerve X associated fibers as they pathfind to their target visceral organs (curved open arrows);

Figures 7E is an 11.5 day anti-Nuk whole-mount showing localization of Nuk protein in the vagus nerve X associated fibers as they pathfind to their target visceral organs (curved open arrows);

Figure 7F is a whole-mount 10 day embryo showing Nuk protein concentrated within the earliest spinal nerve fibers exiting the neural tube (arrows);

Figure 7G shows a slightly later stage embryo from that shown in

Figure 7F;

Figures 7H shows a transverse section bisecting the rostral spinal cord of an 11 day embryo demonstrating that the darkly stained Nuk-positive fibers shown in Figures 7F and 7G are ventral motor axons (open arrows).

Figures 7I shows a transverse section bisecting the rostral spinal cord of an 11 day embryo demonstrating that the darkly stained Nuk-positive fibers shown in Figures 7F and 7G are ventral motor axons (open arrows).

Figures 7J shows both sides of an 11 day whole-mount embryo demonstrating that Nuk protein is localized within the spinal motor nerves as they elongate to the plexus regions (open arrows);

Figures 7K shows both sides of an 11 day whole-mount embryo demonstrating that Nuk protein is localized within the spinal motor nerves as they elongate to the plexus regions;

Figure 7L shows a close-up of a 12 day whole-mount embryo immunostained with anti-Nuk antibodies which label the nerve fibers of the parasympathetic chain ganglion;

Figure 8A shows a whole-mount 10.5 day embryo showing Nuk protein localization (arrow) at the dorsal region of the otic vesicle (ov) surrounding the budding endolymphatic duct;

Figure 8B shows a whole-mount 11.5 day embryo showing elongation of the endolymphatic duct;

Figure 8C shows a whole-mount 11.5 day embryo at a slightly different focal plane from Figure 8B showing that the vestibulocochlear sensory fibers connecting to the developing ear stain positive for Nuk protein (arrow);

Figure 8D shows a transverse section of an 11.5 day embryo showing high levels of Nuk protein localized to the basement membrane of the endolymphatic duct cells where they contact the surrounding mesenchymal cells (open arrow;

Figure 9 shows a recombinant DNA molecule of the invention having a null mutation obtained by deletion of exon 2, corresponding to codons 29 to 50 as shown in SEQ ID NO: 1;

Figure 10 shows a recombinant DNA molecule of the invention encoding the *Nuk*[1] mutation in the ATP binding region of the kinase domain of Nuk protein, and a lac Z reporter gene;

Figure 11A shows expression of the *Nuk*[2] mutation in a mouse embryo at the six somite stage (8.25 days development) in the brain and developing heart;

Figure 11B shows expression of the *Nuk*[2] mutation in a mouse embryo at the 14 somite stage (8.75 days development) in the hindbrain rhombomeres, the midbrain, diencephalon and in the heart (B)

Figure 12A shows *Nuk*[2] expression in a 10.5 day old mouse embryo in the ventral midbrain, dienchephalon and retinal cells;

Figure 12B shows *Nuk*[2] expression in a 10.5 day old mouse embryo in the brain and spinal cord;

Figure 13 shows an immunoblot illustrating that Nuk protein autophosphorylation is induced by Elk-ligand stimulation;

Figure 14 is an immunoblot showing expression of Nuk protein in cell lines;

Figure 15 is an immunoblot showing binding of phosphorylated/non-phosphorylated Nuk protein to SH2-containing GST-fusion proteins;

Figure 16 is an immunoblot showing binding of phosphorylated/non-phosphorylated Nuk protein to SH2-containing GST-fusion proteins;

Figure 17 is an immunoblot showing binding of Nuk Protein to SH2-containing GST-fusion proteins in the presence of a competing phosphorylated peptide;

Figure 18 shows immunoblots illustrating the phosphorylation of proteins after ELK-ligand stimulation of COS cells.

DETAILED DESCRIPTION OF THE INVENTION

I. Characterization of Nucleic Acid Molecules and Proteins of the Invention

As hereinbefore mentioned, the present inventors have identified and sequenced a nucleic acid molecule encoding a novel receptor tyrosine

kinase protein with a unique expression pattern as described herein. The receptor tyrosine kinase protein of the invention is also referred to herein as Neural Kinase (Nuk) protein.

The *Nuk* coding region was cloned using a λgt10 expression library constructed from mouse embryo mRNA. The library was probed with a partial n Q1 *Nuk* cDNA insert. Additional 5'*Nuk* coding sequences were obtained by rapid amplification of cDNA ends (RACE). Translation of combined RACE and cDNA clones revealed a single open reading frame of 994 codons.

The *Nuk* locus mapped to the distal end of mouse chromosome 4 near the *ahd*-1 mutation. The Nuk protein belongs to the *Eph/Elk/Eck* family, of which many members are expressed in the developing nervous system. The protein encoded by the deduced amino acid sequence of Nuk has all the hallmarks of an Eph family member, including a number of conserved residues of the Eph family, for example the 20 cysteine residues whose position is conserved in the extracelluar domain of Eph family members (bold type, Figure 2), an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III); between Nuk residues 330-420 and 444-534. The Ig-like domain of Nuk protein contains specific residues (Cys^{70}, Trp^{80}, Cys^{115}) known to be conserved in the Ig superfamily (Williams and Barclay, *Ann. Rev. Immunol.* 6:381-405, 1988). When compared to other known members of the Eph family, Nuk protein was found to be most highly related to the full length amino acid sequence of chicken Cek5 (96% identity).

In accordance with an embodiment of the invention a purified and isolated nucleic acid molecule is provided containing a sequence encoding a protein having the amino acid sequence as shown in SEQ ID NO:2 and Figure 2. Most preferably, the purified and isolated nucleic acid molecule of the invention contains a nucleic acid sequence as shown in SEQ ID NO:1 and Figure 1.

Fragments of the nucleic acid molecules are contemplated by the present invention. In a preferred embodiment, the fragments include fragments of the nucleotide sequence as shown in SEQ. ID. NO. 1 and in

Figure 1 that have at least 15 bases to 18 bases, preferably at least 15 bases, and which are capable of hybridizing to the nucleotide sequence as shown in SEQ ID NO. 1 and Figure 1 under stringent hybridization conditions as described herein. These fragments may encode, for example, the extracellular domain (amino acids 26 to 548, SEQ ID NO:2) or the carboxy tail (amino acids 601 to 994, SEQ ID NO:2).

It will also be appreciated that a double stranded nucleotide sequence comprising a nucleic acid molecule of the invention or a fragment thereof, hydrogen bonded to a complementary nucleotide base sequence, and an RNA made by transcription of this double stranded nucleotide sequence are contemplated by the present invention.

Further, it will be appreciated that the invention includes nucleic acid or amino acid sequences which have substantial sequence identity with the nucleic acid and amino acid sequences shown in SEQ ID NOS:1 and 2 and in Figures 1 and 2, and fragments thereof. The term "sequences having substantial sequence identity" means those nucleic acid and amino acid sequences which have slight or inconsequential sequence variations from the sequences disclosed in Figures 1 and 2 and SEQ ID NOS: 1 and 2, i.e. the homologous sequences function in substantially the same manner to produce substantially the same polypeptides as the actual sequences. The variations may be attributable to local mutations or structural modifications.

Nucleic acid sequences having substantial identity include nucleic acid sequences which encode proteins having at least 97% sequence identity with the amino acid sequences as shown in SEQ. ID. NO:2 and in Figure 2; nucleic acid sequences having at least 85%, preferably at least 90%, most preferably at least 95% identity with the nucleic acid sequence as shown in SEQ. ID. NO.:1 and in Figure 1; and fragments thereof having at least 15 to 18, preferably at least 15 bases which will hybridize to these sequences under stringent hybridization conditions. Stringent hybridization conditions are those which are stringent enough to provide specificity, reduce the number of mismatches and yet are sufficiently flexible to allow formation of stable hybrids at an acceptable rate. Such conditions are known to those skilled in the

art and are described, for example, in Sambrook, et al, (1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor). By way of example only, stringent hybridization with short nucleotides may be carried out at 5-10° below the T_m using high concentrations of probe such as 0.01-1.0pmole/ml.

The invention further provides amino acid sequences which have substantial identity with the amino acid sequence shown in SEQ ID NO:2 and in Figure 2. Substantially identical sequences include sequences having at least 97% sequence identity. The invention still further provides peptides which are unique to the receptor tyrosine kinase protein of the invention. Preferably, the peptides have at least 10 to 20 amino acids.

The sequence of the nucleic acid molecule of the invention or a fragment thereof, may be inverted relative to its normal presentation for transcription to produce antisense nucleic acid molecules. The antisense nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid molecules may be used in gene therapy to treat inherited disorders of the nervous system.

A number of unique restriction sequences for restriction enzymes are incorporated in the nucleic acid sequence identified in SEQ ID NO: 1 and in Figure 1 and these provide access to nucleic acid sequences which code for polypeptides unique to the receptor tyrosine kinase protein of the invention. Nucleic acid sequences unique to the receptor tyrosine kinase protein of the invention or isoforms or parts thereof, can also be constructed by chemical synthesis and enzymatic ligation reactions carried out by procedures known in the art.

The invention contemplates isoforms of the receptor tyrosine kinase protein of the invention. An isoform contains the same number and kinds of amino acids as the protein of the invention, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are those having the same functional properties as the novel receptor tyrosine kinase protein of the invention as described herein.

The present invention also includes conjugates of the receptor

tyrosine kinase protein of the invention, or parts thereof. For example, the receptor tyrosine kinase protein or portions thereof may be conjugated with a selected protein or marker protein to produce fusion proteins.

The present invention also includes a receptor tyrosine kinase protein of the invention or part thereof, preferably the catalytic domain, which is enzymatically active. The catalytically active form of the protein or part thereof is also referred to herein as an "activated receptor tyrosine kinase protein or part thereof".

II. Expression Pattern of the Receptor Tyrosine Kinase Protein of the Invention

The expression of Nuk protein has been localized during early embryogenesis. The restricted expression of Nuk imposes constraints on the cellular range of activity of the putative Nuk ligand, and indicates that the *Nuk* locus plays unique and important roles in the determination, migration and pathfinding of axons, in axogenesis and fasciculation, in neural tube formation, and in the regulation of specific cell-cell interactions during early development of the nervous system. As many features of regeneration in the peripheral nervous system recapitulate development, for example, following injury and wallerian degeneration, axons regrow and migrate to reinervate their targets, the *Nuk* locus also plays an important role in axonal migration during regeneration following injury to the peripheral nervous system.

The present inventors have shown that, in the early stages of embryonic development, Nuk protein is confined to the developing nervous system, where it marks segments along the axis of the neural tube in the hindbrain and specific morphological bulges of the midbrain and forebrain. Nuk is expressed in a rhombomere-specific pattern early during hindbrain segmentation. The restriction of Nuk protein to specific anterior-posterior and dorsal-ventral compartments during early development of the rostral neural tube indicates this receptor tyrosine kinase protein functions in the patterning of specific brain structures.

Nuk protein was found to be expressed in the developing nervous system and, in particular, is highly expressed very early in the retinal ganglion

cells and in the group of cells that form the optic chiasm just prior to axonogenesis of the retinal cells. These observations indicate that Nuk protein participates in early development of the visual system components and in pathfinding of retinal axons.

The present inventors have also detected Nuk in cells of the ventral midbrain and in the endolymphatic duct of the developing ear.

The present inventors have also localized Nuk protein to specific locations within the cells of the developing nervous system and have shown that Nuk protein is associated with the plasma membrane of migrating neural cells. In particular, Nuk protein is concentrated at sites of cell-cell contact, of migrating neuronal cells or their extensions and high levels of Nuk protein are found within initial axon outgrowths and associated nerve fibers. The axonal localization of Nuk is transient and is not detected after the growth cones have reached their targets and migrations have ceased, indicating a role for this receptor tyrosine kinase protein during the early migration, pathfinding and fasciculation stages of axonogenesis. Nuk protein may function to transmit signals from the plasma membrane and may cooperate with other neuronal tyrosine kinases, such as Sek.

The present inventors found that Nuk protein is localized to at least one CNS axon pathway. The subcellular localization of Nuk protein is similar to that observed for vimentin and the extracelluar matrix molecule laminin (Liesi, *EMBO* 4:1163-1170, 1985) and it coincides with pathways of neuronal cell migration along the radial glial fibers (Hatten, *Trends Neurosci.* 13:179-184, 1990).

The specific subcellular localization of Nuk protein to the cell-cell contacts between the basement membrane of the endolymphatic duct cells and the surrounding mesenchyme/neural crest cells indicates Nuk protein functions to modulate this interaction.

The concentration of Nuk protein at sites of cell-cell contact indicates that its ligand is a membrane-associated molecule. In addition, Nuk protein immunoreactivity was frequently observed on the membranes of both cells at the site of contact and was generally observed to localize to specific regions of

the membrane (see Figures 5 and 8). Therefore, homophilic/heterophilic interactions between Eph receptors may play a role in their biological functions.

III. Preparation of Nucleic Acid Molecules and Proteins of the Invention

The nucleic acid molecules of the invention encoding the novel receptor tyrosine kinase protein, or fragments thereof, may be isolated and sequenced, for example, by synthesizing cDNAs from mouse embryo RNA and using rapid amplification of cDNA ends (RACE, Frohman, et al., Proc. Na+, Acad. Sci. USA, 85, 8998-9002, 1988) using oligonucleotides specific for the novel receptor tyrosine kinase protein, and analysing the sequences of the clones obtained following amplification. Oligonucleotides specific for the novel receptor tyrosine kinase protein may be identified by comparing the nucleic acid sequence of the nucleic acid molecules of the invention to known sequences, for example, sequences of the other members of the *Eph* subfamily. Nucleic acid molecules of the present invention encoding the novel receptor tyrosine kinase protein and oligonucleotide fragments thereof, may also be constructed by chemical synthesis and enzymatic ligation reactions using procedures known in the art.

The novel tyrosine kinase receptor protein of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention having a sequence which codes for the receptor tyrosine kinase protein of the invention, or a fragment thereof may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein or part thereof. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses, so long as the vector is compatible with the host cell used.

The invention therefore contemplates a recombinant molecule of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary elements for the transcription and translation of the inserted protein-sequence. Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate

transcription and translation elements is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other genetic elements, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary transcriptional and translation elements may be supplied by the native receptor tyrosine kinase protein and/or its flanking regions.

The recombinant molecules of the invention may also contain a reporter gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of reporter genes are genes encoding a protein such as β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. In a preferred embodiment, the reporter gene is *lac Z*. Transcription of the reporter gene is monitored by changes in the concentration of the reporter protein such as β-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. This makes it possible to visualize and assay for expression of recombinant molecules of the invention and in particular to determine the effect of a mutation on expression and phenotype.

Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation etc. Methods for transforming transfecting, etc. host cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., PNAS USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition,

Cold Spring Harbor Laboratory Press, 1989, all of which are incorporated herein by reference and see the detailed discussion below).

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, plant, or insect cells.

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli, B. subtilis, Salmonella typhimurium,* and various species within the genus' *Pseudomonas, Streptomyces,* and *Staphylococcus,* as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples of bacterial host cells include DH5n (Stratagene, LaJolla, California), JM109 ATCC No. 53323, HB101 ATCC No. 33694, and MN294. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β-lactamase (penicillinase) and lactose promoter system (see Chang et al., Nature 275:615, 1978), the trp promoter (Nichols and Yanofsky, Meth in Enzymology 101:155, 1983) and the tac promoter (Russell et al., Gene 20: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (see Bolivar et al., Gene 2:9S, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, Meth in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.).

Yeast and fungi host cells suitable for carrying out the present invention include, among others *Saccharomyces cerevisae,* the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus.* Suitable expression vectors for yeast and fungi include, among others, YC_p50 (ATCC No. 37419) for yeast, and the amdS cloning vector pV3 (Turnbull, Bio/Technology 7:169, 1989). Protocols for the transformation of yeast are also well known to those of ordinary skill in the art. For example, transformation

may be readily accomplished either by preparation of spheroplasts of yeast with DNA (see Hinnen et al., PNAS USA 75:1929, 1978) or by treatment with alkaline salts such as LiCl (see Itoh et al., J. Bacteriology 153:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (Bio/Technology 5:369, 1987).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcriptional and translational control sequences. Common promoters include SV40, MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR. Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated electroporation, retroviral, and protoplast fusion-mediated transfection (see Sambrook et al., supra).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, *Nuk* or derivatives thereof may be expressed from plant cells (see Sinkar et al., J. Biosci (Bangalore) 11:47-58, 1987, which reviews the use of Agrobacterium rhizogenes vectors; see also Zambryski et al., Genetic Engineering, Principles and Methods, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York, 1984, which describes the use of expression vectors for plant cells, including, among others, pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx* or *Spodotera* species. Suitable expression vectors for directing expression in insect cells include Baculoviruses such as the *Autographa california* nuclear polyhedrosis, virus (Miller et al. 1987, in *Genetic Engineering*, Vol. 8 ed. Setler, J.K. et al., Plenum Press, New York) and

the *Bombyx mori* nuclear polyhedrosis virus (Maeda et al., 1985, Nature 315:592).

Alternatively, *Nuk* may be expressed in non-human transgenic animals such as, mice, rats, rabbits, sheep and pigs (see Hammer et al. (Nature 315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866).

The Nuk protein or parts thereof may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

Conjugates of the Nuk protein of the invention, or parts thereof, with other molecules, such as proteins or polypeptides, may be prepared. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins. Thus, fusion proteins may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of Nuk protein or parts thereof, and the sequence of a selected protein or marker protein with a desired biological function. The resultant fusion proteins contain Nuk protein or a portion thereof fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins and parts thereof such as the constant region of immunglobulin γ1, and lymphokines such as gamma interferon, tumor necrosis factor, IL-1, IL-2,IL-3, Il-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, GM-CSF, CSF-1 and G-CSF.

Sequences which encode the above-described proteins may generally be obtained from a variety of sources, including for example, depositories which contain plasmids encoding sequences including the American Type Culture Collection (ATCC, Rockville Maryland), and the British Biotechnology Limited (Cowley, Oxford England). Examples of such plasmids include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma

interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon,) ATCC Nos. 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No. 67024 (which contains a sequence which encodes Interleukin-1β), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC Nos. 57592 (which contains sequences encoding Interleukin-4). ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Within a particularly preferred embodiment of the invention, *Nuk* is cloned into an expression vector as a fusion gene with the constant region of human immunoglobulin γ1. Briefly, the expression vectors pNUTΔGH and pVL1393 are prepared for cloning by digestion with SmaI followed by dephosphorylation by calf intestinal alkaline phosphatase. The linear product is isolated after agarose gel electrophoresis. The *Nuk* genes are then generated by polymerase chain reaction using the cloned *Nuk* cDNA as a template. In particular, the Nuk fusion protein is synthesized from the extracelluar domain of Nuk protein, preferably amino acids 26 to 548, SEQ ID NO: 2 and Figure 2. In another embodiment, the Nuk fusion protein is synthesized from the carboxy terminal tail of Nuk protein, preferably amino acids 601 to 994, SEQ ID NO:2 and Figure 2.

The constant region of an immunoglobulin, such as human γ1 gene may be prepared, for example, from pUCB7Ig monomer. Briefly, the C_H gene is isolated by digestion with XbaI which cuts at the 3' end of the gene followed by treatment with *E. coli* DNA polymerase I in the presence of all four dNTPs in order to create a blunt end. The plasmid is then digested with BclI which cuts at the 5' end of the gene. The fragment containing the heavy chain gene is isolated after electrophoresis in an agarose gel.

The fusion *Nuk* amplified fragment is inserted into each prepared vector along with the heavy chain fragment. Orientation of the resulting

plasmids is determined by PCR with one priming oligo which anneals to the vector sequence and the other priming oligo which anneals to the insert sequence. Alternatively, appropriate restriction digests can be performed to verify the orientation. The sequence of the fusion *Nuk*/immunoglobulin constant region gene can be verified by DNA sequencing.

Phosphorylated receptor tyrosine kinase proteins of the invention, or parts thereof, may be prepared using the method described in Reedijk et al. The EMBO Journal 11(4):1365, 1992. For example, tyrosine phosphorylation may be induced by infecting bacteria harbouring a plasmid containing a nucleotide sequence of the invention or fragment thereof, with a λgt11 bacteriophage encoding the cytoplasmic domain of the Elk tyrosine kinase. Bacteria containing the plasmid and bacteriophage as a lysogen are isolated. Following induction of the lysogen, the expressed receptor protein becomes phosphorylated.

IV. Utility of the Nucleic Acid Molecules and Proteins of the Invention

The nucleic acid molecules of the invention or fragments thereof, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences in biological materials. A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleotide probes may be used to detect genes, preferably in human cells, that encode proteins related to or analogous to the receptor tyrosine kinase protein of the invention. The nucleotide probes may therefore be useful in the diagnosis of disorders of the

nervous system arising from mutations or alterations to the *Nuk* gene or a homologue thereof.

The receptor tyrosine kinase protein of the invention and portions thereof, for example amino acids of the carboxy terminal tail, preferably amino acids 601 to 994; or amino acids of the extracellular domain, preferably amino acids 26 to 548 (SEQ ID NO: 2 and Figure 2), may be used to prepare antibodies. Antibodies having specificity for Nuk protein may also be raised from fusion proteins created by expressing trpE-Nuk fusion proteins in bacteria as described above.

Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and $F(ab')_2$ and recombinantly produced binding partners. Antibodies are understood to be reactive against Nuk protein if they bind with a K_a of greater than or equal to 10^{-7} M. As will be appreciated by one of ordinary skill in the art, antibodies may be developed which not only bind to Nuk protein, but which bind to a ligand of Nuk protein, and which also block the biological activity of Nuk protein. Such antibodies will be useful in the diagnosis and treatment of disorders of the nervous system and nerve damage.

Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Briefly, Nuk protein is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous injections, in conjunction with an adjuvant such as Freund's complete or incomplete adjuvant. Following several booster immunizations, samples of serum are collected and tested for reactivity to Nuk protein. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to Nuk protein, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using

conventional techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

Briefly, within one embodiment a subject animal such as a rat or mouse is injected with Nuk protein. The Nuk protein may be admixed with an adjuvant such as Freund's complete or incomplete adjuvant in order to increase the resultant immune response. Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization, and tested for reactivity to Nuk protein using assays described above. Once the animal has plateaued in its reactivity to Nuk protein, it is sacrificed, and organs which contain large numbers of B cells such as the spleen and lymph nodes are harvested.

Cells which are obtained from the immunized animal may be immortalized by transfection with a virus such as the Epstein bar virus (EBV) (see Glasky and Reading, Hybridoma 8(4):377-389, 1989). Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibody. Suitable myeloma lines include, for example, NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580).

Following the fusion, the cells may be placed into culture plates containing a suitable medium, such as RPMI 1640, or DMEM (Dulbecco's Modified Eagles Medium) (JRH Biosciences, Lenexa, Kansas), as well as additional ingredients, such as Fetal Bovine Serum (FBS, ie., from Hyclone, Logan, Utah, or JRH Biosciences). Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells such as HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Missouri). After about seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of

antibodies which are reactive against Nuk protein. A wide variety of assays may be utilized to determine the presence of antibodies which are reactive against Nuk protein, including for example Countercurrent Immuno-Electrophoresis, Radioimmunoassays, Radioimmunoprecipitations, Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition Assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,186,530; see also Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Following several clonal dilutions and reassays, a hybridoma producing antibodies reactive against Nuk protein may be isolated.

Other techniques may also be utilized to construct monoclonal antibodies (see William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, December 1989; see also L. Sastry et al., "Cloning of the Immunological Repertoire in Escherichia coli for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," Proc Natl. Acad. Sci USA 86:5728-5732, August 1989; see also Michelle Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," Strategies in Molecular Biology 3:1-9, January 1990; these references describe a commercial system available from Stratacyte, La Jolla, California, which enables the production of antibodies through recombinant techniques). Briefly, mRNA is isolated from a B cell population, and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the λImmunoZap(H) and λImmunoZap(L) vectors. These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (see Huse et al. supra; see also Sastry et al., supra). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from E. coli.

Similarly, binding partners may also be constructed utilizing recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a

monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Primers for mouse and human variable regions including, among others, primers for V_{Ha}, V_{Hb}, V_{Hc}, V_{Hd}, C_{H1}, V_L and C_L regions are available from Stratacyte (La Jolla, Calif). These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAP™H or ImmunoZAP™L (Stratacyte), respectively. These vectors may then be introduced into E. coli for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the VH and VL domains may be produced (See Bird et al., Science 242:423-426, 1988). In addition, such techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.

Once suitable antibodies or binding partners have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

The polyclonal or monoclonal antibodies may be used to detect the receptor tyrosine kinase protein of the invention in various biological materials, for example they may be used in an ELISA, radioimmunoassay or histochemical tests. Thus, the antibodies may be used to quantify the amount of a receptor tyrosine kinase protein of the invention in a sample in order to determine its role in particular cellular events or pathological states and to diagnose and treat such pathological states.

In particular, the polyclonal and monoclonal antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect the novel receptor tyrosine kinase protein of the invention, to localise it to particular cells and tissues and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens

using light and electron microscopy may be used to detect the novel tyrosine kinase of the invention. Generally, an antibody of the invention may be labelled with a detectable substance and the novel receptor tyrosine kinase of the invention may be localised in tissue based upon the presence of the detectable substance. Examples of detectable substances include various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive iodine I^{125}, I^{131} or tritium. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

Radioactive labelled materials may be prepared by radiolabeling with ^{125}I by the chloramine-T method (Greenwood et al, Biochem. J. 89:114, 1963), the lactoperoxidase method (Marchalonis et al, Biochem. J. 124:921, 1971), the Bolton-Hunter method (Bolton and Hunter, Biochem. J. 133:529, 1973 and Bolton Review 18, Amersham International Limited, Buckinghamshire, England, 1977), the iodogen method (Fraker and Speck, Biochem. Biophys. Res. Commun. 80:849, 1978), the Iodo-beads method (Markwell Anal. Biochem. 125:427, 1982) or with tritium by reductive methylation (Tack et al., J. Biol. Chem. 255:8842, 1980).

Known coupling methods (for example Wilson and Nakane, in "Immunofluorescence and Related Staining Techniques", W. Knapp et al, eds, p. 215, Elsevier/North-Holland, Amsterdam & New York, 1978; P. Tijssen and E. Kurstak, Anal. Biochem. 136:451, 1984) may be used to prepare enzyme labelled materials. Fluorescent labelled materials may be prepared by reacting the material with umbelliferone, fluorescein, fluorescein isothiocyanate, dichlorotriazinylamine fluorescein, dansyl chloride, derivatives of rhodamine such as tetramethyl rhodamine isothiocyanate, or phycoerythrin.

Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against the novel tyrosine kinase of the invention. By way of example, if the antibody having specificity against the novel tyrosine kinase protein of the invention is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, the novel tyrosine kinase of the invention may be localized by radioautography. The results of radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting the grains.

As discussed above, the expression patterns found for the novel tyrosine kinase of the invention indicate that it plays unique and important roles in the determination, migration and pathfinding of axons, in axogenesis and fasciculation, development and regeneration of the neural tube, and in the regulation of specific cell-cell interactions during early development of the nervous system. Therefore, the above described methods for detecting nucleic acid molecules and fragments thereof and Nuk protein and parts thereof, can be used to monitor vertebrate axonal migration, fasciculation and regeneration by detecting and localizing the novel tyrosine kinase protein of the invention in migrating axons and in the migrating membrane surface of cells of the developing nervous system.

It would also be apparent to one skilled in the art that the above described methods may be used to study the developmental expression of *Nuk* and, accordingly, will provide further insight into the role of Nuk protein in neuronal cell:cell interactions in embryogenic development and in axonogenesis and nerve regeneration.

The finding of a novel receptor tyrosine kinase protein which is expressed in migrating axons and the developing neural tube permits the identification of substances which may affect axonogenesis, neural embryonic development and neuron regeneration. A substance which affects expression

of Nuk protein may be assayed using the above described methods for detecting nucleic acid molecules and fragments thereof and Nuk protein and parts thereof, by comparing the pattern and level of expression of the Nuk protein or parts thereof, in the presence and absence of the substance.

The invention also provides methods for identifying substances which are capable of binding to the Nuk protein, or isoforms and parts thereof. In particular, the methods may be used to identify ligands and natural and synthetic derivatives of such ligands, which are capable of binding to and in some cases activating the receptor tyrosine kinase protein of the invention, isoforms thereof, or part of the protein.

Substances which can bind with the receptor tyrosine kinase protein of the invention may be identified by reacting the novel receptor tyrosine kinase protein which is expressed in migrating vertebrate axons isoforms thereof, or part of the protein, with a substance which potentially binds to the novel receptor tyrosine kinase protein, isoforms thereof, or part of the protein such as the extracelluar domain, and assaying for substance-receptor complexes, for free substance or for non-complexed receptor tyrosine kinase protein isoforms thereof or part of the protein, or for activation of the receptor tyrosine kinase protein.

Conditions which permit the formation of substance-receptor protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the receptor protein.

The substance-receptor complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against the receptor protein or the substance, or a labelled receptor protein, or a labelled substance may be utilized. Antibodies, receptor protein or substance may be labelled with a detectable substance as described above.

The receptor tyrosine kinase protein, isoforms or parts thereof, or substance used in the method of the invention may be insolubilized. For

example, the receptor protein or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The insolubilized receptor tyrosine kinase protein or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The receptor tyrosine kinase protein, parts thereof, or substance may also be expressed on the surface of a cell using the methods described herein.

The above mentioned methods of the invention may be used to identify ligands which bind with and activate the novel receptor tyrosine kinase protein of the invention thereby affecting signalling pathways, particularly those involved in neuronal development and axonal migration and regeneration. Identification and isolation of such a Nuk protein ligand will permit studies of the role of the ligand in the developmental regulation of axonogenesis and neural regeneration, and permit the development of substances which affect these roles, such as functional or non-functional analogues of the ligand. It will be appreciated that such substances will be useful as pharmaceuticals to modulate axonogenesis, nerve cell interactions and regeneration to treat conditions such as neurodegenerative diseases and cases of nerve injury.

Ligands which bind to and activate the novel receptor tyrosine kinase protein of the invention may be identified by assaying for protein tyrosine kinase activity i.e. by assaying for phosphorylation of the tyrosine residues of the novel receptor tyrosine kinase protein.

Receptor tyrosine kinase protein activity may be assayed using known techniques such as those using anti-phosphotyrosine antibodies and labelled phosphorous. For example, immunoblots of the complexes may be

analyzed by autoradiography (^{32}P-labelled samples) or may be blocked and probed with antiphosphotyrosine antibodies as described in Koch, C.A. et al (1989) Mol. Cell. Biol. 9, 4131-4140.

The ligands for many receptor tyrosine kinase proteins are cell-bound, either as they are associated with the cell surface via heparin and hepatocyte growth factor or because they are transmembrane proteins (Lyman et al. 1993, supra). Ligands for receptor tyrosine kinases of the *Eph/Elk/Eck* subfamily of receptor tyrosine kinases may require cell-to-cell contact to activate the receptor. Membrane attachment of the ligand could facilitate ligand dimerization or clustering, or both, which may promote receptor multimerization and activation.

Accordingly, a ligand for Nuk protein may have a cell-bound form. A cell-bound ligand may be identified by reacting the receptor tyrosine kinase protein of the invention, an isoform or a part thereof with a cell suspected of expressing the ligand on the surface of the cell following the procedures generally described in Lyman et al., 1993, (Cell 75:1157-1167). Thus, the invention provides a method for identifying cells expressing a surface bound ligand of Nuk protein and for specifically selecting for such cells.

By way of example, a cDNA encoding a ligand for Nuk protein may be cloned by first constructing a fusion protein. The fusion protein may consist of the extracelluar domain of Nuk protein (amino acids 26 to 548, SEQ ID NO: 2 and Figure 2). The fusion protein may be expressed and used as a probe to examine cells or cell lines (e.g. neuroblastoma or neuroepitheliomia cell lines) for their capacity to bind the extracellular domain of Nuk protein (determined by flow cytometry). The identification of cells and cell lines that bind the extracellular domain may be facilitated by incorporating in the fusion protein a sequence encoding a marker protein for example, the Fc portion of human IgG which may be detected with labelled anti-human IgG antibodies. Cells or cell lines which bind the extracellular domain are presumed to express a cell-bound form of the ligand.

Following identification of a source of the Nuk ligand, a cDNA expression library is constructed, following known techniques, using mRNA

from the cells/cell lines which have been identified as binding the fusion protein containing the extracellular domain of Nuk protein. cDNAs are then transfected into host cells (e.g. COS cells and see discussion herein re host cells) which are then screened for their capacity to bind the extracellular domain of Nuk protein. Individual clones which are capable of binding the extracellular domain of Nuk protein are identified and the cDNAs are sequenced. The cDNAs may be used as hybridization probes to isolate genomic DNA encoding the ligand.

The invention also contemplates a method for assaying for an agonist or antagonist of the binding of the novel receptor tyrosine kinase of the invention with a substance which is capable of binding with the novel tyrosine kinase protein, preferably a ligand. The agonist or antagonist may be an endogenous physiological substance or it may be a natural or synthetic drug. Substances which are capable of binding with the Nuk protein and preferably ligands, including cell-bound ligands may be identified using the methods set forth herein. Substances which bind to other receptor tyrosine kinases of the *Eph/Elk/Eck* subfamily may also be used in this assay. For example, the substance may be LERK-2, a binding protein for the receptor-tyrosine kinase ELK (Fletcher, F.A. et al., Oncogene (1994), 9, 3241-3247), or the cell-bound ligands B61 (also known as EFL-1), EHK1-L (also known as EFL-2) and ELK-L (also known as EFL-3)(Davis, S. et al., Science Vol. 266, p.816, Nov. 4, 1994).

In accordance with a preferred embodiment, a method is provided which comprises providing a known concentration of the novel receptor tyrosine kinase protein of the invention, incubating the protein with a ligand which can bind to and activate the protein, and a suspected agonist or antagonist under conditions which permit the formation of substance-receptor protein complexes, and assaying for substance-receptor protein complexes, for free substance, for non-complexed proteins, or for activation of the receptor tyrosine kinase protein. Conditions which permit the formation of substance-receptor protein complexes, and methods for assaying for substance-receptor protein complexes, for free substance, for non-complexed

proteins, or for activation of the receptor tyrosine kinase protein are described herein.

It will be understood that the agonists and antagonists that can be assayed using the methods of the invention may act on one or more of the binding sites on the receptor tyrosine kinase or the ligand, including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of Nuk protein with a Nuk protein ligand. Thus, the invention may be used to assay for a substance that competes for the same ligand binding site of the novel receptor tyrosine kinase protein of the invention.

The invention further contemplates a method for identifying a substance which is capable of binding to an activated receptor tyrosine kinase protein of the invention or an isoform or part of the activated protein, comprising reacting an activated receptor tyrosine kinase protein of the invention, or an isoform, or part of the protein, with at least one substance which potentially can bind with the receptor tyrosine kinase protein, isoform or part of the protein, under conditions which permit the formation of substance-receptor kinase protein complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for phosphorylation of the substance.

An activated receptor tyrosine kinase protein of the invention, or isoform or part thereof may be prepared by binding of a ligand to the extracellular domain of a receptor tyrosine kinase protein of the invention which results in activation of the catalytic domain. Such a ligand may be identified using the methods hereinbefore described. An activated receptor or part thereof, may also be prepared using the methods described for example in Reedijk et al. The EMBO Journal, 11(4):1365, 1992 for producing a tyrosine phosphorylated receptor or part thereof.

Conditions which permit the formation of substance-receptor

protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the receptor protein. The substance-receptor complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques described above. Phosphorylation of the substance may be determined using for example, labelled phosphorous as described above.

In an embodiment of this method, intracellular ligands such as Src homology region 2 (SH2)-containing proteins which are capable of binding to a phosphorylated activated receptor tyrosine kinase protein of the invention may be identified. SH2-containing proteins refers to proteins containing a Src homology region 2 which is a noncatalytic domain of ~100 amino acids which was originally identified in the Vfps and Vsrc cytoplasmic tyrosine kinases by virtue of its effects on both catalytic activity and substrate phosphorylation (T. Pawson, Oncogene 3, 491 (1988) and I. Sadowski et al., Mol. Cell. Biol. 6, 4396 (1986)). (See also Koch et al., Science 252:668, 1991; Moran et al., PNAS USA 87:8622 and Anderson et al., Science 250:979, 1990 for discussions on SH2-containing proteins and the role of SH2 domains). SH2-containing proteins may function downstream of the Nuk signalling pathway by binding to the activated receptor protein. For example, the cytoplasmic tyrosine kinases of the Src family may bind via their SH2 domains to the activated Nuk receptor protein thereby regulating cellular processes particularly in the nervous system. Intracellular ligands which may be phosphorylated by the novel receptor tyrosine kinase of the invention may also be identified using the method of the invention.

SH2-domains of cytoplasmic signalling proteins have been found to bind to the phosphorylated receptor tyrosine kinase protein of the invention. In particular, the SH2 domains of $p21^{ras}$ GTPase-activating protein (GAP), Src, and phosphoinositide-specific phospholipase C (PLCγ) have been found to bind Nuk protein. The SH-2 domain binding site on the Nuk protein is a conserved tyrosine containing region which is located adjacent to the membrane and it corresponds to amino acids 600 to 618 as shown in SEQ. ID. NO:2 in the Sequence Listing.

Therefore, the invention also contemplates a method for assaying for an agonist or antagonist of the binding of an activated receptor tyrosine kinase of the invention, or a portion thereof with an SH2 domain of an intracellular ligand. The agonist or antagonist may be an endogenous physiological substance or it may be a natural or synthetic drug. The activated receptor may be prepared as described herein or a portion of the ligand comprising the amino acid sequence 600 to 618 as shown in SEQ. ID. NO:2 in the Sequence Listing may be used in this method of the invention. Examples of SH2 domains of intracellular ligands include the SH2 domains of GAP, Src, and PLCγ. It will be appreciated that the entire intracellular ligand may be used in this method.

It has also been found that COS cells express large amounts of Nuk protein. Accordingly, COS cells may be used to identify *in vivo*, intracellular proteins or ligands which bind to the Nuk protein.

The invention further provides a method for assaying for a substance that affects axonal migration, neural development, nerve cell interactions and nerve regeneration comprising administering to a non-human animal or to a tissue of an animal, a substance suspected of affecting axonal migration, and detecting, and optionally quantitating, the novel receptor tyrosine kinase of the invention in the non-human animal or tissue. In an embodiment of the invention, the method may be used to assay for a substance that affects axonal migration during embryogenesis. The novel receptor tyrosine kinase of the invention may be quantitated using the methods described herein.

In another embodiment, the method may be used to assay for a substance that affects axonal migration in nerve regeneration, comprising administering a substance suspected of affecting axonal migration to a non-human animal having an injured peripheral nervous system and detecting, and optionally quantitating, the novel receptor tyrosine kinase of the invention in the non-human animal. Examples of non-human animals having an injured peripheral nervous system include animals having damaged axons, such as axotomized facial neurons (Sendtner et al. Nature,

345, 440-441, 1990), neurodegenerative conditions (for example, the MPTP model as described in Langston J.W. et al., Symposium of Current Concepts and Controversies in Parkinson's Disease, Montebello, Quebec, Canada, 1983 and Tatton W.G. et al., Can. J. Neurol. Sci. 1992, 19), and traumatic and non-traumatic peripheral nerve damage (for example, animal stroke models such as the one described in MacMillan et al. Brain Research 151:353-368 (1978)).

Substances which are capable of binding to the Nuk protein of the invention or parts thereof, particularly ligands, and agonists and antagonists of the binding of ligands and Nuk protein, identified by the methods of the invention, may be used for stimulating or inhibiting neuronal development, regeneration and axonal migration. The ligands, agonists and antagonists may accordingly be used to stimulate or inhibit neuronal development, regeneration and axonal migration associated with neurodegenerative conditions and conditions involving trauma and injury to the nervous system, for example Alzheimer's disease, Parkinson's disease, Huntington's disease, demylinating diseases, such as multiple sclerosis, amyotrophic lateral sclerosis, bacterial and viral infections of the nervous system, deficiency diseases, such as Wernicke's disease and nutritional polyneuropathy, progressive supranuclear palsy, Shy Drager's syndrome, multistem degeneration and olivo ponto cerebellar atrophy, peripheral nerve damage, trauma and ischemia resulting from stroke.

The invention also provides methods for studying the function of the Nuk protein. Cells, tissues, and non-human animals lacking in *Nuk* expression or partially lacking in *Nuk* expression may be developed using recombinant molecules of the invention having specific deletion or insertion mutations in the *Nuk* gene. For example, the extracellular domain or parts thereof, such as the FN III and Ig domains; the transmembrane region or parts thereof; the tyrosine kinase domain or parts thereof, such as the ATP binding site and; the carboxy terminal tail may be deleted. A recombinant molecule may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create a *Nuk* deficient cell, tissue or animal.

Null alleles may be generated in cells, such as embryonic stem cells by deletion mutation. A recombinant *Nuk* gene may also be engineered to contain an insertion mutation which inactivates *Nuk*. Such a construct may then be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, electroporation, injection etc. Cells lacking an intact *Nuk* gene may then be identified, for example by Southern blotting, Northern Blotting or by assaying for expression of Nuk protein using the methods described herein. Such cells may then be fused to embryonic stem cells to generate transgenic non-human animals deficient in *Nuk*. Germline transmission of the mutation may be achieved, for example, by aggregating the embryonic stem cells with early stage embryos, such as 8 cell embryos, *in vitro*; transferring the resulting blastocysts into recipient females and; generating germline transmission of the resulting aggregation chimeras. Such a mutant animal may be used to define specific nerve cell populations, developmental patterns of axonogenesis, neural tube formation and nerve regeneration and *in vivo* processes, normally dependent on *Nuk* expression.

The present inventors have generated a loss of function deletion mutation in *Nuk*, designated Nuk^1 in mouse embryonic stem cells, and have achieved germline transmission of this null allele. In particular, the *Nuk* mutation was obtained by deletion of exon 2, corresponding to codons 29 to 50, as shown in Figure 9. Adult animals homozygous for the mutation did not produce any Nuk protein.

A second targeted mutation, designated Nuk^2 was generated in the *Nuk* gene as shown in Figure 10 using the *pPNT-LOX-*Nuk^2 gene trap vector to delete the GXGXXG ATP binding region of the kinase domain (amino acids 623-707, SEQ ID NO:2 and Figure 2) and to create a Nuk-lac Z fusion receptor. Chimeric animals expressing Nuk^2 were prepared. Animals generated with the Nuk^2 mutation provided Nuk^2 expressing cells staining for β-galactosidase activity, providing a convenient marker for *Nuk*-positive cells in both heterozygous and homozygous backgrounds as detected by a blue/green colour as shown in Figures 11 and 12.

The invention also provides methods for preparing cells, tissues,

and non-human animals lacking in *Nuk* expression or partially lacking in *Nuk* expression, and deficient in the expression of other genes. In accordance with one embodiment, an animal may be generated which is deficient in Nuk and another tyrosine kinase of the *Eph/Elk/Eck* subfamily. Such animals could be used to determine how the members of the *Eph/Elk/Eck* subfamily co-operate in embryonic development, particularly development of the nervous system. For example, an animal lacking or partially lacking Nuk expression and Sek-4 expression (Becker N., et al., Mechanisms of Development 47 (1994) 3-17) may be generated to determine how the receptor tyrosine kinases co-operate in the segmental patterning of the hindbrain.

Multiple deficient mice can also be generated to study the interaction of Nuk protein and other proteins such as the Src-family of cytoplasmic tyrosine kinases. For example, an animal may be generated which lacks or partially lacks *Nuk* expression, and expression of one or more *Src* family tyrosine kinases including *Src, Fyn,* and/or *Yes*.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

The following materials and methods were utilized in the investigations outlined in the examples:

cDNA cloning, sequencing and chromosomal *mapping*

To clone the *Nuk* coding region, the partial λQ1 *Nuk* cDNA insert described in Ben-David *et al.*, 1991 (EMBO 10:317) was used to probe a λgt10 cDNA library constructed from 12.5 day mouse embryo mRNA (Logan *et al.*, *Dev. Genet.* 13:345-358, 1992). Sequence analysis of a number of independent clones revealed that none reached as far as the ATG translation initiation codon. To obtain additional 5' *Nuk* coding sequences, rapid amplification of cDNA ends (RACE; Frohman *et al.*, Proc. Nat. Acad. Sci. USA 85:8998-9002, 1988) was performed using *Nuk*-specific oligonucleotides. 9.5 day embryonic poly(A)+ RNA was used for the RACE because Northern blot analysis determined this to be a source of abundant 4.0 and 4.5 Kb *Nuk* mRNA

transcripts. A number of *Nuk* cDNA clones were generated with this approach, however, a strong stop in the majority of the RACE products just 5' of an NheI restriction site prevented cloning of the complete coding region. One clone out of over 50 examined (pNukRACE 2.15) extended far enough such that an oligonucleotide could be used to colony screen further RACE products. Using this approach two independent clones out of over 1000 screened (pNukRACE A2 and K2) were both found to contain an additional 67 base pairs of *Nuk* coding sequences 5' of the NheI site. Translation of combined RACE and cDNA clones revealed a single open reading frame of 993 codons. Although lacking an ATG translation initiation codon, the sequence of the RACE clones encode a 26 amino acid hydrophobic signal peptide. The 5'-most sequence of both RACE clones end with the dinucleotide TG. Given that the length of the Nuk signal peptide is longer than most Eph family members, this TG may represent the last two nucleotides of the ATG initiator codon.

The DNAs generated in this study were subcloned into either the pGEM7 (Promega) or pCRII (Invitrogen) plasmid vectors prior to double stranded sequencing using the Sequenase system (United States Biochemical Corporation). Sequencing reactions were primed using either the standard forward and reverse primers or custom oligonucleotides synthesized in house on a Pharmacia Gene Assembler Plus.

Chromosome mapping was performed by probing Pst I restriction endonulcease digested DNAs from 28 different recombinant inbred mouse strains at high stringency with a 1.0 Kb EcoRI fragment of the λQ1 *Nuk* cDNA.

Generation of antibodies

A trpE-Nuk fusion protein was created by subcloning a NcoI-BamHI fragment of λQ1 containing the carboxy terminal 94 *Nuk* codons plus 170 additional nucleotides of 3' untranslated sequences into the bacterial expression vector pATH1. A GST-Nuk fusion protein containing Nuk amino acids 601-994 was also expressed in bacteria. The trpE-Nuk fusion protein was purified from induced bacterial cultures by SDS-PAGE and used to immunize

rabbits. Resulting trpE-Nuk antiserum was affinity-purified by binding to immobilized glutathione agarose purified GST-Nuk.

Immunoprecipitation, in vitro kinase, and western blotting

For biochemical studies, natural matings of CD1 mice were used to obtain embryos at 10.5, 12.5, and 14.5 days development. The embryos were collected and washed twice in phosphate buffer saline (PBS; 150 mM NaCl, 3 mM KCl, 9 mM $Na_2HPO_4 \cdot 2H_2O$, and 2 mM KH_2PO_4) prior to homogenization in PLC-lysis buffer on ice (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaPPi, 100 mM NaF, 10 µg/ml aprotinin and leupeptin, 1 mM Na_3VO_4, and 1 mM PMSF). The embryo lysates were clarified by centrifugation at 12,000 x rpm for 10 min at 4°C. The cleared supernatant was put on ice and an aliquot was used for protein assay (BCA protein assay, PIERCE). For immunoprecipitations, 1mg of total protein in 1 ml PLC-lysis buffer were incubated with approximately 4 mg of affinity-purified anti-Nuk antibody and 100 ml of a 10% solution of protein A-sepharose beads. Preimmune serum, as well as preincubation of the anti-Nuk antibodies with a competing trpE-Nuk peptide, were used as controls in the immunoprecipitations. After 1 hour incubation at 4°C, the immunoprecipitates were collected by centrifugation at 12,000 x rpm for 30sec and washed three times with ice cold HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 1 mM Na_3VO_4).

For *in vitro* kinase assays, the washed immunoprecipitates were incubated at room temperature (RT) for 15 minutes in 20 ml of kinase buffer (20 mM HEPES, pH7.5, 25 mM $MgCl_2$, 4 mM $MnCl_2$, and 0.1 mM Na_3VO_4) containing 10 µCi of γ^{32}P-ATP (Dupont; 3000 Ci/mmole^{-1}). The immune complex kinase reaction products were denatured at 100°C for 5 min in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Gels were fixed in acetic acid, submerged in 1 M KOH at 55°C for 45 min to remove phosphoserine and phosphothreonine, refixed, dried, and then exposed to Kodak XAR film.

For Western blotting, the washed immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and electro-transferred to

a nylon filter using a semi-dry protein blotting apparatus. The filter was blocked overnight at 4°C in PBS containing 5% dry milk prior to incubation in the same solution containing 1 µg/ml anti-Nuk antibodies for 1hr at room temperature (RT). The filter was washed at RT 3 X10 min with TBSN (20 mM TrisHCl, pH 7.5, 150 mM NaCl, and 0.05% NP-40). Nuk immunoreactivity was detected by using affinity-purified goat anti-rabbit horseradish peroxidase (BioRad, 1:20,000 dilution) and Enhanced Chemi-Luminescence reagents (Amersham). The filters were exposed at RT to Kodak XRP-5 film for 30 sec to 5 min.

Whole-Mount Immunohistochemistry

The immunohistochemical detection used in this study was based on the Vectastain ABC Elite-peroxidase system and Vectastain biotin/avidin blocking reagents (Vector Laboratories). The specificity of the anti-Nuk antibody staining was confirmed by a variety of control experiments including those in which the primary antibody was either omitted or preabsorbed with a trpE-Nuk peptide. In addition, other antibodies including a monoclonal antibody raised against the 160 kD subunit of Neurofilament (anti-NF; AMAC Inc.) and a rabbit polyclonal antibody raised against the murine Engrailed (En) proteins were used to control for the specificity of the immunohistochemistry. All observations reported here have been derived from a number of independent experiments all of which gave similar results. The total number of anti-Nuk stained embryos observed at each stage was from 40 to over 100.

Embryos used in this study were obtained from natural matings of CD1 mice and at the required stages of development were dissected and membranes were removed in ice cold 0.1 M phosphate buffer (pH 7.4). Embryos were then fixed with occasional mixing for 2 h on ice in fresh 4% paraformaldhyde in 0.1 M phosphate buffer. In some instances 0.2 to 1% glutaraldhyde was also added to the fixative. The fixative was washed out overnight in 0.1 M phosphate buffer at 4°C prior to gently dehydrating the embryos in a methanol/phosphate buffered saline (PBS) series (15%/85%; 30/70; 50/50; 75/25% H_2O; 100% methanol) on ice for 30 minutes each step. Once in 100% methanol the embryos can be stored at -20°C for at least 2

months. Prior to rehydration and further manipulation, the embryos were transferred into 80% methanol/20% H_2O_2 for 4-6 h at RT to bleach embryos and inactivate endogenous peroxidases. After washing in 100% methanol, the embryos were rehydrated on ice for 30 minutes each with occasional mixing in 75% methanol/25% H_2O, 50% methanol/50% PBS, 30% methanol/70% PBS, 15% methanol/85% PBS, and 100% PBS + 0.01% Triton X-100. The embryos were then gently mixed on a Nutator at RT 2 x 1 h in PBSMT (2% Dry Milk Powder, 0.01% Triton X-100 in PBS). At this step a small number of embryos were placed at 4°C in PBSMT for preabsorbtion of the biotinylated secondary antibodies as described by the manufacturer (Vector Laboratories). The embryos were then blocked overnight at 4°C on Nutator using the Vectastain ABC Elite and blocking kits in PBSMT+3% normal goat serum (NGS) + 10% avidin blocking reagent. The avidin was then washed out in PBSMT 4 x 1 hour at 4°C followed by 2 x 1 hour at RT. The blocked embryos were then incubated on a Nutator for at least 16 h at 4°C with affinity-purified anti-Nuk antibodies (0.5 to 1.0 mg/ml) in PBSMT containing 3% normal goat serum and 10% biotin blocking agent. Unbound primary antibodies and biotin were washed out in PBSMT 4 x 1 hour at 4°C followed by 2 x 1 hour at RT. The preabsorbed biotinylated secondary antibodies were then added to the embryos and incubation was carried out overnight at 4°C on a Nutator. The secondary antibodies were washed out as described above and the Vectastain ABC elite avidin-biotin-HRP reagent in PBSMT+3% NGS was added and allowed to incubate overnight at 4°C on a Nutator. The ABC elite avidin-biotin-HRP reagent was washed out as described above ending with a final wash in PBT (0.2% BSA, 0.01% Triton X-100 in PBS) at RT. For HRP detection, embryos were incubated in 0.3 mg/ml diaminobenzidine (DAB) in PBT at RT for at least 20 minutes. H_2O_2 was added to 0.03% and the embryos were incubated at RT under a dissecting microscope until color density was sufficient, usually about 1-10 minutes. The color of HRP-DAB reaction product can be changed from an orange to dark purple color by the addition of $NiCl_2$ to 0.5%. After staining, embryos were washed in PBT and dehydrated through a ethanol/PBS series: 30/70, 50/50, 80/20, 100% ethanol for 30 minutes each. For light microscopy,

embryos were cleared in benzyl alcohol:benzyl benzoate (1:2). Photography was carried out with either a Wild M10 macroscope, or a Leitz DMRXE compound microscope using Kodak EPY 64 Tungston slide film or Kodak Ektar 100 print film. Whole-mount mRNA in situ were performed as described in Canton & Rossant, (1992) Development: 116, 357-368.

Paraffin section immunohistochemistry

For the immunohistochemical staining of sectioned embryos, dissected animals were fixed with occasional mixing for 2 h on ice in 4% paraformaldhyde + 1% glutaraldehyde in 0.1 M phosphate buffer. Embryos were then washed in phosphate buffer and dehydrated on ice for 30 minutes each with occasional mixing in 25% ethanol/75% PBS, 50% ethanol/50% PBS, 70% ethanol/30% H_2O prior to storage at 4°C. Before embedding in paraffin, embryos were completely dehydrated in 85% ethanol/15% H_2O and then 100% EtOH. The ethanol was replaced with multiple changes of xylene at RT, and the embryos were then equilibrated to 60°C. The xylene was exchanged with many changes of melted paraffin for 2 to 4 hours at 60°C prior to embedding. Embryos were sectioned at 4 to 6 µm and placed on slides freshly subbed in 1% aminopropyltriethoxysilane. Sections were placed on a slide dryer for 1 hour, allowed to dry overnight at RT, and then partially melted at 55°C for 20 min.

The antibody staining was performed exactly as described in the Vectastain ABC Elite instructions. Anti-Nuk antibodies were used at 4 to 8 µg/ml. For Nuk+peptide control 8 µg/ml of trpE-Nuk fusion protein was added to the anti-Nuk antibodies and preincubated for 1 hour before adding to the sections. Anti-NF monoclonal antibodies were used at 4 µg/ml. In some instances, the HRP reaction was carried out in DAB only. These sections were counterstained in hematoxylin prior to mounting with coverslips. In other sections, the HRP reaction was carried out in DAB+$NiCl_2$ to produce a darker higher contrast product for black and white photography. These sections were not counterstained prior to mounting. Photography was performed on a Leitz DMRXE microscope using Kodak EPY 64 tungsten color slide film or Kodak Technical Pan black and white print film.

Transmission electron microscopy

Since the HRP-DAB reaction product produced in the immunohistochemical staining is electron dense, the subcellular localization of Nuk protein using transmission electron microscopy (EM) of sectioned anti-Nuk protein whole mount stained embryos was performed. Similar ultrastructural immunoperoxidase localization studies of the mammalian embryonic central nervous system has been performed using antibodies directed against the glial fibrillary acid protein (Levitt *et al.*, 1981) or stains specific for the extracelluar matrix (Nakanishi, 1983). Embryos were fixed in 4% paraformaldehyde + 1% glutaraldehyde and then immunoreacted as whole-mounts with anti-Nuk antibodies as described above. The stained embryos were postfixed in OsO_4, dehydrated, and embedded in Spurr resin. Ultrathin sections were observed and photographed on a Philips EM430 transmission electron microscope. To render the HRP reaction product more visible, counterstaining of the ultrathin sections with uranyl acetate and lead citrate was ommitted. To control for fixing conditions and morphology, similar staged and fixed embryos were postfixed in OsO_4 and prepared immediately for EM analysis. Other than a loss of lipids from membranes due to extensive washing, the morphology of the immunoreacted samples was comparable to the controls.

EXAMPLE 1

Cloning and chromosomal location of Nuk

The initial *Nuk* cDNA clone, designated λQ1, was isolated from an unamplified λgt11 expression library constructed from a mouse erythroleukemia cell line by screening with anti-phosphotyrosine antibodies (Ben-David *et al.*, *EMBO* 10:317-325, 1991). Sequence analysis of this clone indicated it was a partial cDNA whose expected translation product was closely related to members of the Eph family of receptor-like tyrosine kinases. Standard cDNA screening of a 12.5 day mouse embryo cDNA library and rapid amplification of cDNA ends (RACE) from 9.5 day embryo mRNA was used to clone the remainder of the *Nuk* coding region (except for the A of the presumptive ATG initiation codon) following the methods described above.

Figure 2 shows that the translation of the combined *Nuk* RACE and cDNA sequences revealed a single open reading frame of 994 amino acids containing both a hydrophobic signal peptide and transmembrane domain (long underlines, Figure 2). All the hallmarks of a receptor tyrosine kinase of the Eph family are found in Nuk protein, including 20 cysteine residues whose position is conserved in the extracellular domain of Eph family members (bold type, Figure 2), an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III; between Nuk amino acids residues 330-420 and 444-534). The Ig-like domain of Nuk contains specific residues (Cys^{70}, Trp^{80}, Cys^{115}) known to be conserved in the Ig superfamily (Williams and Barclay, *Ann. Rev. Immunol.* 6:381-405, 1988). When compared to the Ig-like domains found in other receptor tyrosine kinases (O'Bryan *et al.*, Mol. Cell. Biol. 11:5016, 1991), the Nuk Ig-like domain was found to contain a number of conserved residues (overlines, Figure 2). A repeat involving the Nuk Ig-like domain and residues 239 to 268 is apparent (underlines, Figure 2). Although significantly shorter than a normal Ig domain, residues involved in the Nuk-specific repeat correspond to conserved residues often found in Ig-like domains (residues that are both overlined and underlined in Figure 2). Following the transmembrane domain, the Nuk cytoplasmic region contains a tyrosine kinase catalytic domain (brackets, Figure 2) and a carboxy-terminal tail of 106 residues.

The cartoon in Figure 2 shows the location of the various domains. The carboxy-terminal region used to raise the anti-Nuk antibodies is indicated in Figure 2.

Following a 26 amino acid hydrophobic signal peptide, the Nuk protein extracelluar domain is composed of an Ig-like domain and two FN III repeats. The Nuk protein extracelluar domain also contains 20 cysteines whose position is conserved in the Eph family (Lhotak *et al., Mol. Cell. Biol.* 11:2496-2502, 1991). A hydrophobic transmembrane domain divides the Nuk protein into approximately two halves, a 548 amino acid extracelluar region and a 419 amino acid cytoplasmic region which contains a tyrosine kinase catalytic domain.

The Nuk protein sequence was compared to other known members of the *Eph* family. Nuk was found to be most highly related to the full length amino acid sequence of chicken Cek5 (96% identity; Pasquale, *Cell Regulation* 2:523-534, 1991) and to short PCR products of mRNA from rats (*Tyro* 5; Lai and Lemke, *Neuron* 6:691-704, 1991) and humans (*Erk*; Chan and Watt, *Oncogene* 6:1057-1061 1991). The close identity between *Nuk* and *Cek5* suggest they represent the mammalian and avian orthologs of the same progenitor gene. The absence of full length cDNAs for *Tyro* 5 and *Erk* precludes the determination of whether these sequences correspond to the same or a closely related but different gene.

The chromosomal location of *Nuk* was determined by probing for restriction fragment length polymorphisms (RFLPs) in the DNA of a number of recombinant inbred mouse strains derived from matings between AKR/J and DBA/2J mice (B.A. Taylor, personal communication). The *Nuk* locus mapped to the distal end of mouse chromosome 4 near the *ahd*-1 mutation.

EXAMPLE 2

Nuk tyrosine kinase activity in mouse embryos

To investigate the biological function of Nuk protein, antibodies were raised against a bacterial fusion protein containing the C-terminal 94 amino acids of Nuk (trpE-Nuk). Anti-Nuk protein antibodies were affinity-purified by binding to a bacterial GST-Nuk fusion protein. The specificity of the anti-Nuk protein antibodies was assayed by immunoblotting of bacterial lysates expressing either βgal-Nuk or βgal-Elk carboxy-terminal domains. This experiment demonstrated that the anti-Nuk protein antibodies recognized only the β-gal-Nuk fusion protein. To confirm this result with mammalian protein extracts, Elk protein was immunoprecipitated from a rat brain protein lysate with anti-Elk antibodies. Western blot analysis of the immune-complexes verified that anti-Elk, but not anti-Nuk antibodies could recognize Elk. These experiments demonstrated that the affinity-purified anti-Nuk protein antibodies do not cross-react with the related Elk protein.

To assay for Nuk tyrosine kinase activity *in vitro*, Nuk protein was

immunoprecipitated from 10.5, 12.5, and 14.5 day mouse embryo protein lysates and then incubated in the presence of [γ^{32}P]ATP. The expression and tyrosine kinase activity of Nuk in the embryos is shown in Figure 3.

Anti-Nuk protein antibodies immunoprecipitated a protein-tyrosine kinase of 135 kD, as shown in Figure 3A. A highly phosphorylated protein with a relative mobility of 135 kD was detected in protein extracts of 10.5 day (lane 3, Figure 3A), 12.5 day (lane 4, Figure 3A), and 14.5 day (lane 5, Figure 3A) embryos. The mobility of this protein is consistent with that reported for other Eph family members including Eck, Elk, and Cek5 (Lindberg *et al., Mol. Cell. Biol.* 10:6316-6324, 1990; Lhotak *et al., Mol. Cell. Biol.* 11:2496-2502, 1991; Pasquale *et al., J. Neurosci.* 12:3956-3967, 1992). No kinase signal was observed from 10.5 day embryo lysates when preimmune serum was used (lane 1, Figure 3A) or if the anti-Nuk antibodies were initially preincubated with a trpE-Nuk fusion protein (lane 2, Figure 3A). To enrich for phosphotyrosine, the gel was treated with KOH as described previously herein.

Similar anti-Nuk protein immunoprecipitates were also subjected to Western blot analysis with anti-Nuk antibodies, as shown in Figure 3B. Immunoblotting identified Nuk protein as a 135 kD protein, (Figure 3B). The anti-Nuk protein antibodies detected Nuk protein as a 135 kD protein in 10.5 day (lane 3, Figure 3B), 12.5 day (lane 4, Figure 3B), and 14.5 day (lane 5, Figure 3B) embryos. No signal was detected if the 10.5 day embryo lysate was immunoprecipitated with preimmune serum (lane 1, Figure 3B), or with anti-Nuk antibodies pre-incubated with a trpE-Nuk fusion protein (lane 2, Figure 3B). The abundant low molecular weight signals are due to binding by the goat anti-rabbit secondary antibodies to the anti-Nuk protein antibodies and the IgG in the preimmune serum used in the immunoprecipitation. In agreement with the *in vitro* kinase studies, the Western blot revealed highest levels of the 135 kd Nuk protein in 10.5 day mouse embryos (Figure 3B, lane 3).

EXAMPLE 3

Segmental expression of Nuk

The biochemical data presented above and Northern mRNA analysis

indicated *Nuk* is highly expressed in 9.5 to 10.5 day mouse embryos. To investigate this expression in detail, Nuk protein was visualized in 7.5 to 13 day mouse embryos *in situ* using the anti-Nuk protein antibodies in whole-mount and paraffin section immuno-histochemical techniques. To confirm the protein studies, *Nuk* mRNA *in situs* on whole-mounts (Conlon and Rossant, *Development* 116:357-368, 1992) and frozen sections were also performed with similar staged embryos.

Nuk protein localization in whole-mount and paraffin sections of 7.5 to 10.5 day mouse embryos is shown in Figure 4, comprising whole-mount preparations showing *Nuk* mRNA (Figure 4A) and protein (Figures 4B to 4E) at early postimplantation stages of embryonic development. Unless otherwise stated, in Figure 4, dorsal is *left* and anterior is *up*. Figure 4A represents whole-mount mRNA studies which detected *Nuk* transcripts enriched in the neural ectoderm of an embryo at 7.5 days development (note dorsal surface is *up)*. By the 6 somite stage (8 days) Nuk protein is detected as dark orange horseradishperoxidase (HRP) staining as shown in Figure 4B. This is most apparent in the neural groove where relatively high levels are observed in specific hindbrain rhombomeres and in the midbrain region. The arrowhead in Figure 4B points to rhombomeres r2, r3 that express high levels of Nuk protein. The sinus venosis of the developing circulatory system also stains positive for Nuk. Figure 4C shows an embryo at 12 somite stage (8.75 days) and reveals elevated levels of *Nuk* protein in specific regions of the anterior neural tube. The most anterior structures immunoreactive for Nuk include the ventral diencephalon followed by the ventral mesencephalon/midbrain. Rhombomeres r2, r3, and r5 of the hindbrain are also immunoreactive for Nuk protein. The arrow in Figure 4C points to rhombomere 5. The expression of Nuk was confirmed by performing mRNA *in situs* on similar staged embryos.

Figure 4D shows 24 somite (9.5 day) stage embryos immunoreacted with either anti-Nuk protein antibodies (left) or, as a control, anti-Nuk protein antibodies that were preincubated with a trpE-Nuk fusion protein (right). Nuk protein is most abundant in the ventral midbrain, diencephalon, and optic

stalk. Along the spinal cord, high levels of Nuk protein is detected at the dorsal surface of the neural tube (see Figure 5 for more detail). Note the absence of specific staining in the control embryo. Figure 4E shows whole-mount immunolocalization of Nuk protein in the developing brain of a 9.5 day embryo in greater detail. Nuk protein is most highly expressed in the ventral midbrain encompassing the flexure region. Nuk protein is also detected in the ventral diencephalon, optic chiasm, optic stalk, retinal cells (out of focus), and basal telencephalon. At this stage, Nuk protein is still detected in the hindbrain and is localized to the floorplate (arrow in Figure 4E points to ventral region of rhombomere 2). Note the small patch of Nuk protein now detectable in the lateral region of rhombomere 4 just anterior of the otic vesicle.

Figures 4F to 4J show immunohistochemical detection of Nuk protein in paraffin sections of 9.5 and 10.5 day mouse embryos. Figures 4F and 4G show serial transverse sections of a 9.5 day embryo immunoreacted with anti-Nuk antibodies which detect Nuk protein (brown stain) in the ventral midbrain. Nuk protein is localized to all layers of the neural tube including the proliferative ventricular zone, the internal mantel layer, and the outer marginal layer which is adjacent to the surrounding mesenchyme. Note Nuk protein is detected only in the ventral aspect of the midbrain and its limit of expression marks a specific morphological bulge/constriction of the neural tube that separates ventral from dorsal components (arrowhead).

Figure 4H shows a sagittal section along the midline of a 10.5 day embryo showing Nuk protein (dark stain) concentrated in ventral regions of the midbrain and in the optic chiasm (asterisk) and footplate of the hindbrain (thick arrow). The orientation of this embryo is opposite to the one shown in Figure 4E. Note the appearance of a morphological constriction (arrowhead) which separates the midbrain from the diencephalon. Since this is a section along the midline, the infundibulium or ventral most region of the diencephalon is exposed between the midbrain and optic chiasm. This region of the diencephalon does not express Nuk. Figures 4I and 4J show adjacent transverse sections of a 10.5 day embryo immunoreacted with either anti-Nuk

protein antibodies (4I) or anti-Nuk antibodies preincubated with a trpE-Nuk peptide (4J), and illustrate the specificity of the immunohistochemistry. Staining of Nuk protein at 10.5 days persists in the ventral midbrain and is also detected at lower levels in the floorplate of the hindbrain (arrowhead).

The scale bars shown in Figure 4 represent the following measurements: (4A), 100 μm; (4B and 4C), 120 μm; (4E, 4F, 4G, 4I, 4J), 150 μm; (3H), 300 μm.

As described above, by the six somite stage (8 days) high levels of Nuk protein are detected in the neural ectoderm prior to fusion of the dorsal edges of the neural plate (Figure 4B). Highest levels of Nuk immunoreactivity are detected in the presumptive midbrain and in specific rhombomere segments of the hindbrain. Nuk protein is also detected in the sinus venosis of the developing circulatory system. By the 12 somite stage (8.75 days) the dorsal edges of the neural plate have fused forming the neural tube. High levels of Nuk protein are localized to specific compartments of the neural tube including hindbrain rhombomeres r2, r3, and r5, the ventral midbrain (mesencephalon) encompassing the flexure, and the ventral diencephalon (Figure 4C).

By 9.5 to 10.5 days of development, *Nuk* expression intensifies in specific ventral regions of the developing brain structures (Figures 4D and 4E). In the hindbrain at this stage, Nuk protein becomes concentrated along the floor plate. When anti-Nuk protein antibodies are used to stain transverse sections at the midbrain of 9.5 day (Figures 4F and 4G) and 10.5 day (Figure 4I) embryos, it is apparent that Nuk protein is restricted to the morphological bulge corresponding to the ventral/basal plate of the mesencephalon. Preincubating the anti-Nuk protein antibodies with a Nuk peptide abolished the signal, demonstrating the specificity of the antibody in both whole-mount and paraffin section immunohisto-chemistry (Figure 4D and 4J). Staining of additional transverse sections determined that Nuk protein in the diencephalon of 9 to 10.5 day embryos is also restricted to ventral structures, including the hypothalamus, thalamus, and the optic stalk. Very high levels of Nuk protein were also detected in cells of the primitive optic chiasm at day

10.5. Sagittal sections confirmed that high levels of *Nuk* mRNA and protein (Figure 4H) are detected specifically in ventral cells of the developing brain. Consistent with the *in vitro* kinase and Western analysis, anti-Nuk immunoreactivity in 11.5 to 13.5 day embryos decreases significantly in these brain structures as discussed below.

EXAMPLE 4

Subcellular localization of Nuk

The immunohistochemical reaction product observed for the anti-Nuk antibodies often appears as if the Nuk protein is localized to specific surfaces of the plasma membrane. One clear example of this is at the dorsal surface of the posterior neural tube which contains elevated amounts of the Nuk protein as early as 9.5 days of development (see Figure 4D). The subcellular localization of Nuk protein is shown in more detail in Figure 5.

Figures 5A and 5B are adjacent transverse sections of an 11.5 day embryo at the level of the caudal/posterior spinal cord immunoreacted with either anti-Nuk antibodies (5A) or anti-Nuk antibodies preincubated with a trpE-Nuk peptide (5B). Nuk protein is concentrated in the dorsal region of the neural tube along the basement membrane (arrow). This localization of Nuk protein is observed as early as 9.5 days of development in whole-mount preparations (see Figure 4D). Antibodies to Neurofilament (anti-NF) and Engrailed (anti-En) proteins did not stain the basement membrane demonstrating the specificity of the anti-Nuk antibodies.

Figures 5C and 5D show transmission electron microscopy (EM) immunolocalization of Nuk protein in ventral midbrain cells of a 9.5 day embryo. Immunoperoxidase anti-Nuk stained whole-mount embryos were ultrathin sectioned and observed under EM. Figure 5C shows that, at the proliferative ventricular zone (VZ), Nuk-positive signals are concentrated at sites of cell-cell contact (arrows). Notice that Nuk protein immunostaining is often localized on the membranes of both cells that are making contact. At this stage of development there is no obvious ultrastructural differences between neuronal and glial cell types. Therefore, it cannot be distinguished if Nuk protein is associated with neurons, glial, or both cell types. Note the condensed

chromatin evident in the nucleus of a cell that is in late prophase (asterisk).

Figure 5D shows that cells within the postmitotic mantal region of the neural tube also exhibit Nuk protein localization at sites of cell-cell contact. The photomicrograph shows a site of contact between two cells labelled strongly for Nuk protein (filled arrow). Note that other sites of cell-cell contact do not contain detectable amounts of Nuk protein (open arrows).

The scale bars in Figure 5 represent the following measurements: (A and B) 100 μm; (C), 2 μm; (D), 1 μm.

Transverse sections of 11.5 day embryos shows in greater detail that Nuk protein is concentrated where the dorsal-most membrane of the neural tube make contact with the surrounding mesenchyme/neural crest cells (Figures 5A and B). Lateral and ventral regions of the neural tube do not exhibit this localized Nuk protein.

Since the HRP-DAB reaction product produced in the immunohistochemical staining is electron dense, the subcellular localization of Nuk protein was investigated using transmission electron microscopy (EM), following the methods described above. The ventral midbrain of 9.5 day embryos was chosen for EM analysis because of its intense Nuk immunoreactivity. Figure 5C shows a section of the midbrain at the mitotically active ventricular zone. Nuk immuno-reactivity associated with these cells is usually localized to the plasma membranes at specific sites of cell-cell contact. As shown in Figure 5D, Nuk immunoreactivity was not found to be localized to all sites of cell-cell contact. In addition, small punctate deposits throughout the ventral midbrain were immunoreactive for Nuk protein. These structures appear to be plasma membrane material lost from post-mitotic cells during their radial migration from the ventricular zone.

EXAMPLE 5

Nuk localization in pioneer cranial PNS axons and an early CNS axon tract and in spinal axons

Axonogenesis in mouse embryos commences at approximately 10 days of development when neurons associated with both the central and peripheral nervous systems extend axon projections toward their targets.

Anti-Nuk antibody staining of 10 to 12 day embryos revealed that Nuk protein is highly concentrated within most if not all early axonal projections of the PNS and to at least one early pathway of the CNS.

An example of Nuk protein localization in a specific cranial nerve, the oculomotor nerve (III), is shown in Figure 6. Figure 6A is a photomicrograph of the head of an 11 day anti-Nuk whole-mount immunostained embryo with one of the pair of oculomotor nerve fibers in focus showing strong labelling of Nuk protein (filled arrow). A clearer view of Nuk protein staining in the oculomotor axons fibers can be obtained when the whole-mount staged embryo was filleted down the midline to minimize tissue thickness (Figure 6B). This view shows that the Nuk protein positive oculomotor III axons exit the neural tube from the ventral aspect of the midbrain. An anti-Nuk stained frontal section shows that these axons originate from the Nuk-expressing cells in the ventral midbrain, at a region that is consistent with the position of the oculomotor nuclei (Figure 6E). Anti-Nuk antibodies also label an early pathway of the CNS (open arrow in Figure 6).

Figures 6C to 6F show paraffin section immunohistochemistry of 10.5 to 11 day mouse embryos. Adjacent transverse sections bisecting the pair of oculomotor nerve fibers were immunostained with either anti-Nuk (6C) or anti-Neurofilament (6D) antibodies. The arrows in both Figures 6C and 6D point to the darkly stained axon bundles of the two oculomotor nerves. To verify the specificity of the immunohistochemistry, additional control experiments were performed in which the anti-Nuk antibodies were either omitted, preincubated with a trpE-Nuk peptide, or substituted with antibodies directed against the Engrailed (anti-En) nuclear homeodomain proteins. Figure 6E is a frontal section immunostained with anti-Nuk antibodies which label the ventral midbrain and oculomotor axon fibers as they exit the neural tube (filled arrow) and extend (open arrow) towards their target tissue, the pre-optic muscle mass.

Figure 6F shows a sagittal section immunostained with anti-Nuk antibodies which label the oculomoter axon fascicule as it enters the pre-optic

muscle mass (open arrow). This section also shows the expression of Nuk protein in the developing retinal cells (r). The orientation of this section is the same as Figure 4H.

The scale bar for Figures 6 (A) is 180 µm; (B) 120 µm ; and (C-F) 100 µm.

To confirm that the observed Nuk protein localization is in axons, adjacent transverse sections that bisect the oculomotor nerve fibers of a 10.5 day embryo were immunoreacted with either anti-Nuk (Figure 6C) or neuron-specific anti-Neurofilament antibodies (anti-NF; Figure 6D). Both antibodies labelled the same structure demonstrating the axon-specific localization of Nuk protein. The main target tissue of oculomotor axons is the premuscle mass of cells adjacent to the developing retina, whose function in the adult is to control certain eye movements. An anti-Nuk stained sagittal section labelled the oculomotor axon fascicule at the point of entry into the premuscle mass (Figure 6F). This section also shows the high level of Nuk protein associated with the developing retinal cells.

Other cranial PNS axons contain high levels of Nuk protein early during their elongation. Nuk protein is detected in the trigeminal (V) and facial (VII) nerve fibers in 10.5 day embryos (Figures. 7A to 7C). The vagus (X) parasympathetic autonomic nerve, and the accessory (XI) and hypoglossal (XII) somatomotor nerves also contain localized Nuk protein (Figures. 7D and 7E). As observed by the anti-Nuk labelling, these fibers enter a common region, the cardiac/pulmonary plexus, where they then elongate to their targets such as the cardiac muscle and other visceral organs (vagus) or the upper torso (accessory) and tongue (hypoglossal). Nuk protein localization in these cranial axons is very transient and is not detected after 12.5 days development.

The embryo in Figure 6A and B also exhibits specific anti-Nuk labelling in the developing CNS of a connection between the telencephalon and the midbrain. Information describing the naming and position of early tracts in the developing mammalian forebrain is sparce. The location of Nuk immunoreactivity is consistent with the location of axonal projections originating from the ventral midbrain tegmentum such as those of the red

nuclei or the reticular activating system (Carpenter, 1985, Core Text on Neuroanatomy, Baltimore: Williams & Wilkins). Alternatively, this pathway may correspond to the telencephalic/supraoptic tract described in zebra (Chitnis and Kuwanda, J. Neurosci. 10, 1892-1905, 1990; Wilson et al., (1990) Development, 108, 121-143, 1990). Closer definition of the origin and termination sites as well double labelling with other antibody probes should help determine the identity of this Nuk-positive tract.

EXAMPLE 6

Nuk Localization in Spinal Axons

Figures 7A to 7E show Nuk protein localization in cranial nerves of 10.5 to 12 day mouse embryos. Figure 7A is a 10.5 day whole-mount embryo immunostained with anti-Nuk antibodies which label the trigeminal nerve V and facial nerves VII. Figures 7B and 7C show transverse sections of a 10.5 day embryo showing Nuk-positive trigeminal nerve V axon fascicules labelled with the anti-Nuk antibodies. Note that Nuk protein is also present throughout the caudal hindbrain region of the neural tube. Figures 7D and E are 11.5 day anti-Nuk whole-mounts showing localization of Nuk protein in the vagus nerve (X) associated fibers as they pathfind to their target visceral organs (curved open arrows). Other cranial axons including the accessory (XI) and hypoglossal (XII) nerve fibers are immunoreactive for Nuk protein. These cranial nerves initially extend to the plexus region (open arrow in D) before they pathfind to the heart and other target tissues.

Figures 7F to 7L show Nuk protein localization in spinal nerves of 10 to 12 day embryos. Figure 7F is a whole-mount 10 day embryo showing Nuk protein concentrated within the earliest spinal nerve fibers exiting the neural tube (arrows). Figure 7G shows a slightly later stage embryo from that shown in Figure 7F. Notice that the nerve fibers exiting the neural tube have thickened due to fasciculation of additional axons. The insert is a close-up of a single spinal nerve showing Nuk protein is localized throughout its length and can be observed at the leading tips of the growth cones. Figures 7H and 7I show transverse sections bisecting the rostral spinal cord of an 11 day embryo

demonstrating that the darkly stained Nuk-positive fibers shown in Figures 7F and 7G are ventral motor axons (open arrows). At this stage, the accesory nerve also stains positive for Nuk protein as evident by the strong labelling of the axon fibers (filled arrows). The uniform expression of Nuk throughout the spinal region of the neural tube is apparent in these sections.

Figures 7J and 7K show both sides of an 11 day whole-mount embryo demonstrating that Nuk protein is localized within the spinal motor nerves as they elongate to the plexus regions (open arrows in Figure 7J). Between the motor fibers, the appearance of Nuk-positive DRG cell bodies and axons can be observed in this embryo (filled arrows in Figure 7K). Note that the Nuk-positive DRG axons are more apparent in the posterior/caudal segments of the spinal cord. Figure 7L shows a close-up of a 12 day whole-mount embryo immunostained with anti-Nuk antibodies which label the nerve fibers of the parasympathetic chain ganglion. Note the ganglia form a chain of interganglionic axonal connections with each other and that each ganglion unit forms connections with two segments of the neural tube.

Scale bars in Figures 7B, 7C, 7H, and 7I represent 200 μm.

Immunostained whole-mount 10-10.5 day embryos revealed high levels of Nuk protein in the earliest spinal motor axons as they exit the neural tube (Figures 7F and G). The close-up photomicrograph in Figure 7G shows that Nuk protein is localized throughout the length of the axons including the growth cones. Transverse sections immunostained with anti-Nuk antibodies confirmed that Nuk protein is highly localized to the ventral motor axons and the axons of the DRG (Figures 7H and I). Note detectable levels of Nuk protein throughout the spinal cord. By 11 days of development (Figures 7J and K) the Nuk-positive nerve fibers have elongated and thickened considerably due to the fasciculation of additional axons along the initial axons which, by this time, have reached the plexus regions (Tosney and Landmesser, *J. Neurosci.* 4:2518-2527, 1984; Tosney and Landmesser, *Dev. Biol.* 109:193-2141985a; Tosney and Landmesser, *J. Neurosci.* 5:2336-2344 1985b; Landmesser and Swain, *Neuron* 8:291-305, 1992). Nuk protein in the rostral DRG cell bodies and their fibers connecting to the neural tube is also detected (Figures 7J and 7K). By 12

days of development only low levels of Nuk immunoreactivity are detected in the motor and DRG axons. At this stage, Nuk protein is detected in axon fibers of the sympathetic chain ganglion (Figure 7L) and nerve fibers surrounding the heart and diaphram regions.

EXAMPLE 7

Nuk protein early in ear development

Very high levels of Nuk protein were found in specific structures of the developing ear and associated vestibulocochlear (VIII) ganglion in 10.5 to 12.5 day embryos as is shown in Figure 8.

As shown in Figure 8, anti-Nuk immunoreacted whole-mount 10.5 day embryo detects Nuk protein localization (arrow) at the dorsal region of the otic vesicle (ov) surrounding the budding endolymphatic duct (Figure 8A). Branchial arches 1 and 2 are indicated. By 11.5 days of development the endolymphatic duct has elongated approximately 200µm dorsally (Figure 8B). Nuk protein is observed to envelope this structure (arrow). Nuk protein localization within cranial nerve VII as it enters the second branchial arch can also be seen in Figure 8B. Figure 8C shows a slightly different focal plane from (B) showing that the vestibulocochlear sensory fibers connecting to the developing ear stain positive for Nuk protein (arrow). Nuk protein associated with the developing eye is also shown in Figure 8C. Transverse section of an 11.5 day embryo detects high levels of Nuk protein localized to the basement membrane of the endolymphatic duct cells where they contact the surrounding mesenchymal/neural crest cells (open arrow, Figure 8D). Nuk protein associated with the vestibulocochlear ganglion is also visible in this section (filled arrow).

The scale bars in Figure 8 represent the following: (8A), 100 µm; (8B and 8C), 200 µm; (8D), 100 µm.

At 10.5 days Nuk immunostaining associated with the ear is first observed at the dorsal region of the otic vesicle surrounding the initial bulge of the endolymphatic duct (Figure 8A). By 11.5 days Nuk immunoreactivity encapsulates the endolymphatic duct during its dorsal elongation (Figure 8B). A different focal plane of the same embryo shows Nuk is also localized to the

vestibulocochlear ganglion and its axon fibers connecting to the developing ear (Figure 8C). Transverse sections detected Nuk protein specifically localized to the basement membrane of the endolymphatic duct cells (Figure 8D). By 12.5 days of development only low levels of Nuk were detected in the endolymphatic duct and the acoustic ganglion.

EXAMPLE 8

Generation of Loss of Function *Nuk* mutant

A loss of function mutation in *Nuk*, designated *Nuk*^{1} was generated in embryonic stem cells, and germline transmission of the null allele was obtained as described in more detail below.

A null allele was generated in mouse embryonic stem cells generallly following the methodology described in Capecchi M.R., Science 244:1288-1292, 1989. The null mutation was obtained by deletion of exon 2, corresponding to codons 29 to 50, as shown in Figure 9. To obtain germ line transmission of the mutation *Nuk*+/- embryonic stem cell lines (ES) were aggregated with 8 cell embryos *in vitro* and the resulting blastocysts were transferred into recipient females. Upon birth, animals chimeric for ES and embryonic stem cells were recovered by scoring for eye pigment and coat colour. Breeding of these "aggregation chimeras" confirmed that the germ line of at least one founder mouse is derived completely from the ES cells. Adult mice homozygous for the mutation did not express Nuk protein.

EXAMPLE 9

Generation of a *Nuk-lac Z* fusion chimeric receptor mutant

A targeted mutation, designated *Nuk*^{2} was generated in the *Nuk* gene as shown in Figure 10. A *pPNT-LOX-Nuk*^{2} gene trap vector was used to delete the GXGXXG ATP binding region of the kinase domain (amino acids 623-707, SEQ ID NO:2 and Figure 2) to create a Nuk-lac Z fusion receptor in ES cells. Chimeric animals were prepared as described above, by aggregating the ES cells with 8 cell CD1 embryos.

Animals generated with the *Nuk*^{2} mutation provided *Nuk* expressing cells staining for β-galactosidase activity, providing a convenient marker for Nuk-positive cells in both heterozygous and homozygous

backgrounds. The *Nuk*^{2} mutation led to the expression of a Nuk-lac Z fusion protein in mouse heterozygous embryos, detected by a blue/green colour as shown in Figures 11 and 12.

Figure 11A shows an embryo at the six somite stage (8.25 days development) expressing the *Nuk*^{2} mutation in the brain and developing heart. Figure 11B shows that at the 14 somite stage (8.75 days development) expression continues in the hindbrain rhombomeres, the midbrain and diencephalon and persists in the heart. Figure 12 shows *Nuk*^{2} expression in a 10.5 day old embryo. Figure 12A illustrates the very high levels of expression in the ventral midbrain, dienchephalon and retinal cells (which are out of focus in the photomicrograph). Figure 12B illustrates expression in the brain and spinal cord.

EXAMPLE 10

Autophosphorylation of Nuk protein by ELK-ligand

Fusion proteins consisting of the extracellular domain of Elk-ligand (Davis, S., et al. Science Vol. 266, Nov. 4, 1994, p.816) linked to the Fc portion of human immunoglobulin G1 were made in COS cells S/N following the methods outlined in Davis et al, 1994, supra. The expressed ligand was aggregated with anti-human Fc antibody and incubated with COS cells for 5 minutes, 15 minutes, 30 minutes or 1 hour. Following incubation, the COS cells were lysed and the lysate was immunoprecipitated with anti-Nuk antibodies. The immunoprecipitates were subjected to Western blotting analysis with anti-phosphotyrosine antibodies. Figure 13 shows the autophosphorylation of Nuk on tyrosine from 15 minutes to one hour following Elk-ligand stimulation. Therefore, Nuk autophosphorylation is induced by stimulation with the Elk-ligand.

EXAMPLE 11

Nuk expression in Cell Lines

Various cell lines were screened for expression of Nuk protein. Cells were homogenized and the homogenate was incubated with anti-Nuk antibody and the immunoprecipitates were collected by centrifugation. The washed immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis

and subjected to Western blotting with anti-Nuk antibodies or anti-phosphotyrosine antibodies. As shown in Figures 14 and in Table 1, COS cells expressed high levels of Nuk protein.

TABLE 1

	MG87	COS	Rat2	PC12*
Nuk protein	+	++++	+	+

(*NB/ can't stimulate with elk-ligand)

EXAMPLE 12

GST-fusion mixing experiments with Nuk in vitro phosphorylation

GST-fusion proteins containing SH2 domains of p85N, p85C, PLCγN, PLCγC, GAPN, Src, Crk, Abl, and Grb2 were made in E. coli. Nuk protein was immunoprecipitated from lysates of COS cells using Nuk antisera, and phosphorylated by incubation with ATP. Bacterial lysates containing the GST fusion proteins were incubated with phosphorylated/non-phosphorylated Nuk protein. Western blots were prepared using anti-GST antibodies. The results are shown in Figures 15 and 16, and they are summarized below in Table 2.

Table 2

Fusion Protein	Nuk	
	Non-phosphorylated	Phosphorylated
GST	-	-
p85N	+/-	+/-

p85C	-	+
PLCγN	-	++
PLCγC	++	++++
GAPN	+	+++
Src	+	++++
Crk	-	+
Abl	-	+
SH2	-	-
Grb2	No	No

As shown in Table 2, phosphorylated Nuk protein bound to fusion proteins containing SH2 domains of PLCγN, PLCγC, Src, and GAPN.

EXAMPLE 13

GST-fusion mixing experiments with Nuk in vitro phosphorylation

The experiments described in Example 12 were also carried out in the presence of a competing phosphorylated peptide-GMKTpYIDPFTpYEDPNEAVR(K) from the Nuk membrane proximal region. In particular, Nuk from COS cells obtained with Nuk antisera was phosphorylated by incubation with ATP. The phosphorylated Nuk was incubated with bacterial lysates from SH2-GST fusion protein expressing bacteria, in the presence or absence of the competing peptide. Western blots for were prepared using anti-GST antibodies. As shown in Figure 17, the Nuk phosphorylated peptide competes *in vitro* with the interaction of GAP, PLCγ, and Src SH2 fusion proteins. Unphosphorylated peptide did not interfere with

the interaction nor did a nonspecific Trk SHC-binding peptide (HIAENPQY$_p$FSD).

EXAMPLE 14

The phosphorylation of proteins after Elk-ligand stimulation in Nuk expressing COS cells was investigated. Anti-phosphotyrosine Western blots were prepared from whole cell lysates of COS cells stimulated with Elk-ligand. The results are shown in Figure 18. Proteins of about 160, 130 (probably Nuk), 58 and 40 KD were found to be phosphorylated on tyrosine after Elk-ligand stimulation.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

The following sequence listings form part of the application.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS:
(A) NAME: Mount Sinai Hospital
(B) STREET: 600 University Avenue
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP):
(G) TELEPHONE NO.: (416) 586-8225
(H) TELEFAX NO.: (416) 586-8844

(A) NAME: Anthony Pawson
(B) STREET: 34 Glenwood Avenue
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): M6P 3C6

(A) NAME: Mark Henkemeyer
(B) STREET: 263 Adelaide Street West, Apt. 503
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): M5T 1K5

(A) NAME: Kenneth Letwin
(B) STREET: 20 Carluke Crescent, Apt. 1010
(C) CITY: Willowdale
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): M2L 2J1

(ii) TITLE OF INVENTION: NOVEL NEURAL KINASE AND RECEPTOR TYROSINE KINASE

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Bereskin & Parr
(B) STREET: 40 King Street West, Box 401
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) ZIP: M5H 3Y2

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT

(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kurdydyk, Linda M
(B) REGISTRATION NUMBER: 34,971
(C) REFERENCE/DOCKET NUMBER: 3153-148

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (416) 364-7311
(B) TELEFAX: (416) 361-1398
(C) TELEX: 06-23115

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3105 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus
(D) DEVELOPMENTAL STAGE: Embryo

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lambda gt10 cDNA library
(B) CLONE: Combined PnUKRACE A2 and K2 AND cDNA clones

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Distal end of chromosome 4
(B) MAP POSITION: near the ahd-1 mutation

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGGAGCCC GGGTCCCCGT TCTGCCCGGG CTGGATGGCT CATTCTGCTG GCTGCTGCTG 60

CTGCCGCTGC TAGCCGCCGT GGAAGAAACC CTGATGGACT CTACGACAGC AACGGCTGAG 120

CTGGGCTGGA TGGTACATCC CCCATCAGGG TGGGAAGAGG TGAGCGGCTA CGACGAGAAC 180

ATGAACACTA TCCGTACCTA CCAGGTGTGC AATGTCTTTG AGTCAAGCCA GAACAACTGG 240

CTGCGGACCA AATTCATCCG GCGCCGTGGC GCCCACCGTA TCCACGTGGA GATGAAGTTC 300

TCGGTGCGTG ACTGCAGCAG CATTCCCAGC GTGCCGGGCT CCTGCAAGGA GACCTTCAAC 360

CTCTACTACT ATGAGGCTGA TTTTGACTTA GCCACCAAAA CCTTTCCCAA CTGGATGGAG 420

AATCCGTGGG TGAAGGTGGA CACCATCGCG GCCGATGAGA GCTTCTCTCA GGTGGACCTG 480

GGTGGCCGCG TCATGAAAAT CAACACTGAG GTGCGAAGCT TCGGTCCTGT GTCCCGCAAT 540

GGTTTCTACC TGGCCTTCCA GGACTACGGC GGCTGTATGT CCCTCATTGC TGTGCGCGTC 600

TTCTACCGGA AGTGCCCCCG AATCATCCAG AATGGTGCCA TCTTCCAGGA GACACTATCG 660

GGGGCTGAGA GCACTTCGCT GGTGGCAGCT CGGGGCAGCT GCATCGCCAA TGCTGAAGAA 720

GTGGACGTGC CCATCAAACT CTACTGTAAC GGGGACGGCG AATGGCTGGT GCCCATCGGT 780

CGCTGCATGT GCAAGGCGGG CTTCGAGGCT GTGGAGAACG GCACCGTCTG CCGAGGTTGT 840

CCATCAGGAA CCTTCAAGGC CAACCAAGGG GACGAAGCCT GCACCCACTG TCCCATCAAC 900

AGCCGCACCA CCTCTGAGGG TGCCACCAAC TGTGTATGCC GCAACGGCTA CTACAGGGCC 960

GACCTGGACC CCTTAGACAT GCCTTGCACA ACCATCCCCT CTGCGCCCCA GGCTGTGATC 1020

TCCAGCGTCA ACGAGACATC CCTCATGCTA GAGTGGACCC CACCCCGAGA CTCCGGGGGT 1080

CGCGAGGATC TTGTTTACAA CATCATCTGC AAGAGCTGTG GCTCCGGCCG GGGCGCATGC 1140

ACGCGCTGCG GGGACAACGT GCAGTACGCG CCCCGCCAGC TGGGCCTGAC TGAGCCGCGC 1200

ATCTACATCA GTGACCTGCT GGCACACACG CAGTACACCT TCGAGATCCA GGCCGTGAAT 1260

GGTGTGACCG ACCAGAGTCC CTTCTCACCT CAGTTCGCCT CTGTGAACAT CACCACCAAC 1320

CAAGCAGCAC CATCGGCCGT GTCCATCATG CACCAGGTGA GCCGCACTGT GGACAGCATC 1380

ACCCTGTCGT GGTCCCAGCC AGACCAGCCC AACGGTGTGA TCCTGGACTA CGAGCTGCAG 1440

TACTATGAGA AGGAGCTCAG TGAGTACAAC GCCACGGCCA TAAAAAGCCC CACCAACACA 1500

GTCACTGTGC AGGGCCTCAA AGCCGGCGCC ATCTATGTCT TCCAGGTGCG GGCACGCACC 1560

GTTGCAGGCT ATGGGCGCTA CAGTGGCAAG ATGTACTTCC AAACCATGAC AGAAGCCGAG 1620

TACCAGACCA GCATCAAGGA AAAGCTACCC CTCATCGTTG GCTCCTCCGC CGCCGGCTTA 1680

GTCTTCCTCA TCGCTGTGGT CGTCATTGCC ATCGTATGTA ACAGACGGGG GTTTGAGCGT 1740

GCCGACTCAG AGTACACGGA CAAGCTACAG CACTACACCA GCGGACACAT GACCCCAGGC 1800

ATGAAGATCT ATATAGATCC TTTCACCTAT GAAGATCCTA ATGAGGCAGT GCGGGAGTTT 1860

GCCAAGGAAA TTGACATCTC CTGTGTCAAG ATTGAGCAGG TGATTGGAGC AGGGGAATTT 1920

GGTGAGGTCT GCAGTGGCCA TTTGAAGCTG CCAGGCAAGA GAGAGATCTT TGTAGCCATC 1980

AAGACCCTCA AGTCAGGATA CACGGAGAAA CAGCGCCGGG ACTTCCTGAG TGAGGCATCC 2040

ATCATGGGCC AGTTCGACCA CCCCAATGTC ATCCATCTGG AAGGGGTTGT CACCAAGAGC 2100

ACACCTGTCA TGATCATCAC TGAATTCATG GAGAATGGAT CTCTGGACTC CTTCCTCCGG 2160

CAAAATGATG GGCAGTTCAC AGTCATCCAA CTGGTGGGCA TGCTGAGGGG CATTGCAGCC 2220

GGCATGAAGT ACCTGGCGGA CATGAACTAC GTGCACCGTG ACCTTGCTGC TCGAAACATC 2280

CTCGTCAACA GTAACCTGGT GTGTAAGGTG TCTGACTTTG GGCTCTCACG CTTCCTGGAG 2340

GATGACACGT CTGACCCCAC CTATACCAGC GCTCTGGGTG GGAAGATCCC CATCCGTTGG 2400

ACGGCACCGG AAGCCATCCA GTACCGGAAA TTCACCTCGG CCAGTGATGT GTGGAGCTAT 2460

GGCATCGTCA TGTGGGAGGT GATGTCCTAC GGGGAACGAC CCTACTGGGA CATGACCAAT 2520

CAAGACGTAA TCAACGCCAT TGAACAGGAC TACAGACTAC CTCCGCCCAT GGACTGCCCT 2580

AGCGCCCTGC ACCAGCTCAT GCTGGACTGC TGGCAGAAGG ACCGCAACCA CCGGCCCAAG 2640

TTCGGCCAGA TTGTCAACAC GCTGGACAAG ATGATCCGAA ACCCCAACAG CCTCAAAGCC 2700

ATGGCACCCC TGTCCTCTGG CATCAACCTG CCACTGCTGG ACCGCACGAT ACCGGACTAC 2760

ACCAGCTTTA ACACAGTGGA TGAGTGGCTA GAGGCCATCA AGATGGGCCA GTACAAGGAG 2820

AGCTTTGCCA ACGCCGGCTT CACCTCTTTC GACGTTGTAT CTCAGATGAT GATGGAGGAC 2880

ATTCTCCGCG TTGGGGTCAC TCTAGCTGGC CACCAGAAAA AAATCCTGAA CAGTATCCAG 2940

GTGATGCGGG CCCAGATGAA CCAGATCCAG TCTGTAGAGG TTTGACATTC GCCTGCCTCG 3000

GTTCTCCTCT TCCTCCACGC CGCCCCTGAG CCCCTACGTC GGTCCCTGCT GCTCTGTCAC 3060

TGCAGGTCAG CACTGCCAGG AGGCCACAGA CAACAGGAAG ACCAA 3105

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 994 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus
(D) DEVELOPMENTAL STAGE: Embryo

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lamda gt10 cDNA library
(B) CLONE: Combined pNukRACE A2 and K2 and cDNA clones

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Distal end of chromosome 4
(B) MAP POSITION: near the ahd-1 mutation

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Ala Arg Val Pro Val Leu Pro Gly Leu Asp Gly Ser Phe Cys
1 5 10 15

Trp Leu Leu Leu Leu Pro Leu Leu Ala Ala Val Glu Glu Thr Leu Met

```
            20                  25                  30

Asp Ser Thr Thr Ala Thr Ala Glu Leu Gly Trp Met Val His Pro Pro
        35                  40                  45

Ser Gly Trp Glu Glu Val Ser Gly Tyr Asp Glu Asn Met Asn Thr Ile
    50                  55                  60

Arg Thr Tyr Gln Val Cys Asn Val Phe Glu Ser Ser Gln Asn Asn Trp
65                  70                  75                  80

Leu Arg Thr Lys Phe Ile Arg Arg Arg Gly Ala His Arg Ile His Val
                85                  90                  95

Glu Met Lys Phe Ser Val Arg Asp Cys Ser Ser Ile Pro Ser Val Pro
            100                 105                 110

Gly Ser Cys Lys Glu Thr Phe Asn Leu Tyr Tyr Tyr Glu Ala Asp Phe
        115                 120                 125

Asp Leu Ala Thr Lys Thr Phe Pro Asn Trp Met Glu Asn Pro Trp Val
    130                 135                 140

Lys Val Asp Thr Ile Ala Ala Asp Glu Ser Phe Ser Gln Val Asp Leu
145                 150                 155                 160

Gly Gly Arg Val Met Lys Ile Asn Thr Glu Val Arg Ser Phe Gly Pro
                165                 170                 175

Val Ser Arg Asn Gly Phe Tyr Leu Ala Phe Gln Asp Tyr Gly Gly Cys
            180                 185                 190

Met Ser Leu Ile Ala Val Arg Val Phe Tyr Arg Lys Cys Pro Arg Ile
        195                 200                 205

Ile Gln Asn Gly Ala Ile Phe Gln Glu Thr Leu Ser Gly Ala Glu Ser
    210                 215                 220

Thr Ser Leu Val Ala Ala Arg Gly Ser Cys Ile Ala Asn Ala Glu Glu
225                 230                 235                 240

Val Asp Val Pro Ile Lys Leu Tyr Cys Asn Gly Asp Gly Glu Trp Leu
                245                 250                 255

Val Pro Ile Gly Arg Cys Met Cys Lys Ala Gly Phe Glu Ala Val Glu
            260                 265                 270

Asn Gly Thr Val Cys Arg Gly Cys Pro Ser Gly Thr Phe Lys Ala Asn
        275                 280                 285

Gln Gly Asp Glu Ala Cys Thr His Cys Pro Ile Asn Ser Arg Thr Thr
    290                 295                 300

Ser Glu Gly Ala Thr Asn Cys Val Cys Arg Asn Gly Tyr Tyr Arg Ala
305                 310                 315                 320
```

Asp Leu Asp Pro Leu Asp Met Pro Cys Thr Thr Ile Pro Ser Ala Pro
325 330 335

Gln Ala Val Ile Ser Ser Val Asn Glu Thr Ser Leu Met Leu Glu Trp
340 345 350

Thr Pro Pro Arg Asp Ser Gly Gly Arg Glu Asp Leu Val Tyr Asn Ile
355 360 365

Ile Cys Lys Ser Cys Gly Ser Gly Arg Gly Ala Cys Thr Arg Cys Gly
370 375 380

Asp Asn Val Gln Tyr Ala Pro Arg Gln Leu Gly Leu Thr Glu Pro Arg
385 390 395 400

Ile Tyr Ile Ser Asp Leu Leu Ala His Thr Gln Tyr Thr Phe Glu Ile
405 410 415

Gln Ala Val Asn Gly Val Thr Asp Gln Ser Pro Phe Ser Pro Gln Phe
420 425 430

Ala Ser Val Asn Ile Thr Thr Asn Gln Ala Ala Pro Ser Ala Val Ser
435 440 445

Ile Met His Gln Val Ser Arg Thr Val Asp Ser Ile Thr Leu Ser Trp
450 455 460

Ser Gln Pro Asp Gln Pro Asn Gly Val Ile Leu Asp Tyr Glu Leu Gln
465 470 475 480

Tyr Tyr Glu Lys Glu Leu Ser Glu Tyr Asn Ala Thr Ala Ile Lys Ser
485 490 495

Pro Thr Asn Thr Val Thr Val Gln Gly Leu Lys Ala Gly Ala Ile Tyr
500 505 510

Val Phe Gln Val Arg Ala Arg Thr Val Ala Gly Tyr Gly Arg Tyr Ser
515 520 525

Gly Lys Met Tyr Phe Gln Thr Met Thr Glu Ala Glu Tyr Gln Thr Ser
530 535 540

Ile Lys Glu Lys Leu Pro Leu Ile Val Gly Ser Ser Ala Ala Gly Leu
545 550 555 560

Val Phe Leu Ile Ala Val Val Val Ile Ala Ile Val Cys Asn Arg Arg
565 570 575

Gly Phe Glu Arg Ala Asp Ser Glu Tyr Thr Asp Lys Leu Gln His Tyr
580 585 590

Thr Ser Gly His Met Thr Pro Gly Met Lys Ile Tyr Ile Asp Pro Phe
595 600 605

Thr Tyr Glu Asp Pro Asn Glu Ala Val Arg Glu Phe Ala Lys Glu Ile
610 615 620

Asp Ile Ser Cys Val Lys Ile Glu Gln Val Ile Gly Ala Gly Glu Phe
625 630 635 640

Gly Glu Val Cys Ser Gly His Leu Lys Leu Pro Gly Lys Arg Glu Ile
645 650 655

Phe Val Ala Ile Lys Thr Leu Lys Ser Gly Tyr Thr Glu Lys Gln Arg
660 665 670

Arg Asp Phe Leu Ser Glu Ala Ser Ile Met Gly Gln Phe Asp His Pro
675 680 685

Asn Val Ile His Leu Glu Gly Val Val Thr Lys Ser Thr Pro Val Met
690 695 700

Ile Ile Thr Glu Phe Met Glu Asn Gly Ser Leu Asp Ser Phe Leu Arg
705 710 715 720

Gln Asn Asp Gly Gln Phe Thr Val Ile Gln Leu Val Gly Met Leu Arg
725 730 735

Gly Ile Ala Ala Gly Met Lys Tyr Leu Ala Asp Met Asn Tyr Val His
740 745 750

Arg Asp Leu Ala Ala Arg Asn Ile Leu Val Asn Ser Asn Leu Val Cys
755 760 765

Lys Val Ser Asp Phe Gly Leu Ser Arg Phe Leu Glu Asp Asp Thr Ser
770 775 780

Asp Pro Thr Tyr Thr Ser Ala Leu Gly Gly Lys Ile Pro Ile Arg Trp
785 790 795 800

Thr Ala Pro Glu Ala Ile Gln Tyr Arg Lys Phe Thr Ser Ala Ser Asp
805 810 815

Val Trp Ser Tyr Gly Ile Val Met Trp Glu Val Met Ser Tyr Gly Glu
820 825 830

Arg Pro Tyr Trp Asp Met Thr Asn Gln Asp Val Ile Asn Ala Ile Glu
835 840 845

Gln Asp Tyr Arg Leu Pro Pro Pro Met Asp Cys Pro Ser Ala Leu His
850 855 860

Gln Leu Met Leu Asp Cys Trp Gln Lys Asp Arg Asn His Arg Pro Lys
865 870 875 880

Phe Gly Gln Ile Val Asn Thr Leu Asp Lys Met Ile Arg Asn Pro Asn
885 890 895

Ser Leu Lys Ala Met Ala Pro Leu Ser Ser Gly Ile Asn Leu Pro Leu
900 905 910

Leu Asp Arg Thr Ile Pro Asp Tyr Thr Ser Phe Asn Thr Val Asp Glu
915 920 925

Trp Leu Glu Ala Ile Lys Met Gly Gln Tyr Lys Glu Ser Phe Ala Asn
930 935 940

Ala Gly Phe Thr Ser Phe Asp Val Val Ser Gln Met Met Met Glu Asp
945 950 955 960

Ile Leu Arg Val Gly Val Thr Leu Ala Gly His Gln Lys Lys Ile Leu
965 970 975

Asn Ser Ile Gln Val Met Arg Ala Gln Met Asn Gln Ile Gln Ser Val
980 985 990

Glu Val

WE CLAIM:

1. An isolated and purified nucleic acid molecule comprising:

(a) a nucleic acid sequence encoding a protein having the amino acid sequence as shown in SEQ ID NO:2 and Figure 2;

(b) nucleic acid sequences complementary to (a);

(c) nucleic acid sequences which are at least 97% homologous to (a); or,

(d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.

2. A purified and isolated nucleic acid molecule as claimed in claim 1 comprising:

(a) a nucleic acid sequence as shown in SEQ ID NO:1 and Figure 1, wherein T can also be U;

(b) nucleic acid sequences complementary to (a);

(c) nucleic acid sequences which are at least 85% homologous to (a); or,

(d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.

3. A purified and isolated receptor tyrosine kinase protein having an amino acid sequence as shown in SEQ ID NO:2 or a sequence having at least 97% homology thereto, or an isoform or a part of the protein having at least 20 amino acids.

4. A part of the protein as claimed in claim 3 comprising an extracellular domain of a tyrosine kinase having the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 26 to 548 or a sequence having at lease 97% homology thereto.

5. A part of the protein as claimed in claim 3 comprising a carboxy

terminal of a tyrosine kinase protein having the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 601 to 994 or a sequence having at lease 97% homology thereto.

6. A recombinant molecule comprising a nucleic acid molecule as claimed in claim 1 and one or more transcription or translation elements operatively linked thereto.

7. A transformant host cell including a recombinant molecule comprising a nucleic acid molecule as claimed in claim 1 and one or more transcription or translation elements operatively linked thereto.

8. A method for preparing a receptor tyrosine kinase protein comprising expressing a purified and isolated nucleic acid molecule as claimed in claim 1 in a host cell.

9. A method for identifying a substance which is capable of binding to a receptor tyrosine kinase protein or an isoform or part thereof as claimed in claim 3 comprising reacting the receptor tyrosine kinase protein or an isoform or part thereof, under conditions which permit the formation of a complex between the substance and the receptor tyrosine kinase protein, or isoform or part thereof, assaying for substance-receptor complexes, for free substance, for non-complexed receptor tyrosine kinase protein, or isoforms or parts thereof, or for activation of the receptor tyrosine kinase protein.

10. The method as claimed in claim 9, wherein the substance is a ligand of the receptor tyrosine kinase protein which is capable of activating the receptor tyrosine kinase.

11. The method as claimed in claim 9 wherein the part of the receptor tyrosine kinase protein has the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 26 to 548 or a sequence having at least 97 %

homology thereto.

12. A method for assaying a medium for the presence of an agonist or antagonist of the interaction of a receptor tyrosine kinase protein as claimed in claim 3 and a substance which is capable of binding to the receptor tyrosine kinase protein, which comprises providing a known concentration of the receptor tyrosine kinase protein, reacting the receptor tyrosine kinase protein with a substance which is capable of binding to the receptor tyrosine kinase protein and a suspected agonist or antagonist under conditions which permit the formation of substance-receptor tyrosine kinase protein complexes, and assaying for substance-receptor tyrosine kinase protein complexes, for free substance, for non-complexed proteins, or for activation of the receptor tyrosine kinase protein.

13. A method for identifying a substance which is capable of binding to an activated receptor tyrosine kinase protein as claimed in claim 3, or isoform or part thereof which is activated comprising reacting a receptor tyrosine kinase protein as claimed in claim 3 which is activated, or an isoform or part of the protein, with at least one substance which potentially can bind with the receptor tyrosine kinase protein, isoform or part of the protein, under conditions which permit the formation of substance-receptor kinase protein complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for phosphorylation of the substance.

14. A monoclonal or polyclonal antibody specific for an epitope of a protein as claimed in claim 3.

15. A recombinant DNA molecule adapted for transformation of a host cell comprising a nucleic acid molecule as claimed in claim 2 wherein a portion of the nucleic acid sequence is deleted, and one or more transcription or translation elements operatively linked thereto.

16. A transgenic non-human mammal all of whose germ cells and somatic cells contain a recombinant DNA molecule as claimed in claim 15 introduced into the animal, or an ancestor of the mammal at an embryonic stage.

FIGURE 1

```
ATGGGAGCCCGGGTCCCCGTTCTGCCCGGGCTGGATGGCTCATTCTGCTGGCTGCTGCTG      60
CTGCCGCTGCTAGCCGCCGTGGAAGAAACCCTGATGGACTCTACGACAGCAACGGCTGAG     120
CTGGGCTGGATGGTACATCCCCCATCAGGGTGGGAAGAGGTGAGCGGCTACGACGAGAAC     180
ATGAACACTATCCGTACCTACCAGGTGTGCAATGTCTTTGAGTCAAGCCAGAACAACTGG     240
CTGCGGACCAAATTCATCCGGCGCCGTGGCGCCCACCGTATCCACGTGGAGATGAAGTTC     300
TCGGTGCGTGACTGCAGCAGCATTCCCAGCGTGCCGGGCTCCTGCAAGGAGACCTTCAAC     360
CTCTACTACTATGAGGCTGATTTTGACTTAGCCACCAAAACCTTTCCCAACTGGATGGAG     420
AATCCGTGGGTGAAGGTGGACACCATCGCGGCCGATGAGAGCTTCTCTCAGGTGGACCTG     480
GGTGGCCGCGTCATGAAAATCAACACTGAGGTGCGAAGCTTCGGTCCTGTGTCCCGCAAT     540
GGTTTCTACCTGGCCTTCCAGGACTACGGCGGCTGTATGTCCCTCATTGCTGTGCGCGTC     600
TTCTACCGGAAGTGCCCCCGAATCATCCAGAATGGTGCCATCTTCCAGGAGACACTATCG     660
GGGGCTGAGAGCACTTCGCTGGTGGCAGCTCGGGGCAGCTGCATCGCCAATGCTGAAGAA     720
GTGGACGTGCCCATCAAACTCTACTGTAACGGGGACGGCGAATGGCTGGTGCCCATCGGT     780
CGCTGCATGTGCAAGGCGGGCTTCGAGGCTGTGGAGAACGGCACCGTCTGCCGAGGTTGT     840
CCATCAGGAACCTTCAAGGCCAACCAAGGGGACGAAGCCTGCACCCACTGTCCCATCAAC     900
AGCCGCACCACCTCTGAGGGTGCCACCAACTGTGTATGCCGCAACGGCTACTACAGGGCC     960
GACCTGGACCCCTTAGACATGCCTTGCACAACCATCCCCTCTGCGCCCCAGGCTGTGATC    1020
TCCAGCGTCAACGAGACATCCCTCATGCTAGAGTGGACCCCACCCCGAGACTCCGGGGGT    1080
CGCGAGGATCTTGTTTACAACATCATCTGCAAGAGCTGTGGCTCCGGCCGGGGCGCATGC    1140
ACGCGCTGCGGGGACAACGTGCAGTACGCGCCCCGCCAGCTGGGCCTGACTGAGCCGCGC    1200
ATCTACATCAGTGACCTGCTGGCACACACGCAGTACACCTTCGAGATCCAGGCCGTGAAT    1260
GGTGTGACCGACCAGAGTCCCTTCTCACCTCAGTTCGCCTCTGTGAACATCACCACCAAC    1320
CAAGCAGCACCATCGGCCGTGTCCATCATGCACCAGGTGAGCCGCACTGTGGACAGCATC    1380
ACCCTGTCGTGGTCCCAGCCAGACCAGCCCAACGGTGTGATCCTGGACTACGAGCTGCAG    1440
TACTATGAGAAGGAGCTCAGTGAGTACAACGCCACGGCCATAAAAAGCCCCACCAACACA    1500
GTCACTGTGCAGGGCCTCAAAGCCGGCGCCATCTATGTCTTCCAGGTGCGGGCACGCACC    1560
```

FIGURE 1 CONT'D

GTTGCAGGCTATGGGCGCTACAGTGGCAAGATGTACTTCCAAACCATGACAGAAGCCGAG 1620

TACCAGACCAGCATCAAGGAAAAGCTACCCCTCATCGTTGGCTCCTCCGCCGCCGGCTTA 1680

GTCTTCCTCATCGCTGTGGTCGTCATTGCCATCGTATGTAACAGACGGGGGTTTGAGCGT 1740

GCCGACTCAGAGTACACGGACAAGCTACAGCACTACACCAGCGGACACATGACCCCAGGC 1800

ATGAAGATCTATATAGATCCTTTCACCTATGAAGATCCTAATGAGGCAGTGCGGGAGTTT 1860

GCCAAGGAAATTGACATCTCCTGTGTCAAGATTGAGCAGGTGATTGGAGCAGGGGAATTT 1920

GGTGAGGTCTGCAGTGGCCATTTGAAGCTGCCAGGCAAGAGAGAGATCTTTGTAGCCATC 1980

AAGACCCTCAAGTCAGGATACACGGAGAAACAGCGCCGGGACTTCCTGAGTGAGGCATCC 2040

ATCATGGGCCAGTTCGACCACCCCAATGTCATCCATCTGGAAGGGGTTGTCACCAAGAGC 2100

ACACCTGTCATGATCATCACTGAATTCATGGAGAATGGATCTCTGGACTCCTTCCTCCGG 2160

CAAAATGATGGGCAGTTCACAGTCATCCAACTGGTGGGCATGCTGAGGGGCATTGCAGCC 2220

GGCATGAAGTACCTGGCGGACATGAACTACGTGCACCGTGACCTTGCTGCTCGAAACATC 2280

CTCGTCAACAGTAACCTGGTGTGTAAGGTGTCTGACTTTGGGCTCTCACGCTTCCTGGAG 2340

GATGACACGTCTGACCCCACCTATACCAGCGCTCTGGGTGGGAAGATCCCCATCCGTTGG 2400

ACGGCACCGGAAGCCATCCAGTACCGGAAATTCACCTCGGCCAGTGATGTGTGGAGCTAT 2460

GGCATCGTCATGTGGGAGGTGATGTCCTACGGGGAACGACCCTACTGGGACATGACCAAT 2520

CAAGACGTAATCAACGCCATTGAACAGGACTACAGACTACCTCCGCCCATGGACTGCCCT 2580

AGCGCCCTGCACCAGCTCATGCTGGACTGCTGGCAGAAGGACCGCAACCACCGGCCCAAG 2640

TTCGGCCAGATTGTCAACACGCTGGACAAGATGATCCGAAACCCCAACAGCCTCAAAGCC 2700

ATGGCACCCCTGTCCTCTGGCATCAACCTGCCACTGCTGGACCGCACGATACCGGACTAC 2760

ACCAGCTTTAACACAGTGGATGAGTGGCTAGAGGCCATCAAGATGGGCCAGTACAAGGAG 2820

AGCTTTGCCAACGCCGGCTTCACCTCTTTCGACGTTGTATCTCAGATGATGATGGAGGAC 2880

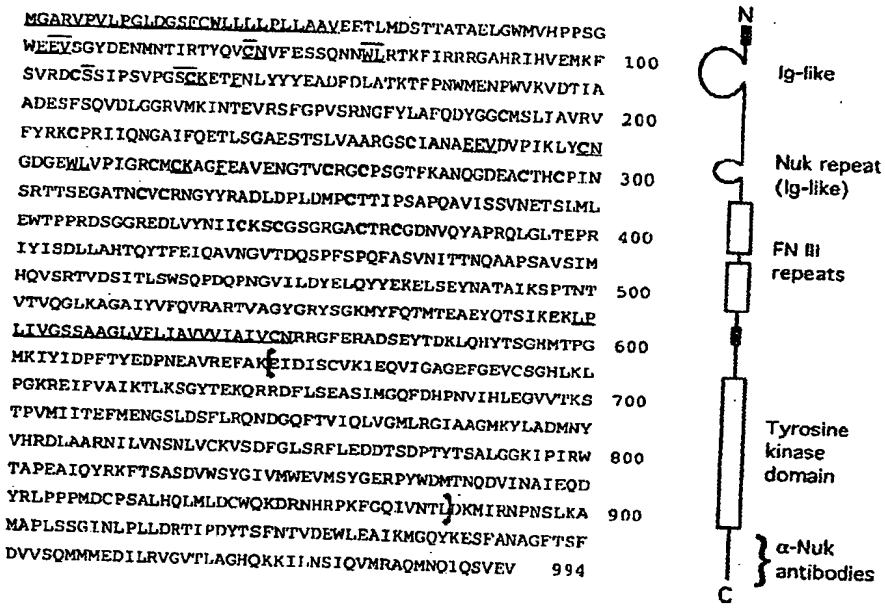
ATTCTCCGCGTTGGGGTCACTCTAGCTGGCCACCAGAAAAAAATCCTGAACAGTATCCAG 2940

GTGATGCGGGCCCAGATGAACCAGATCCAGTCTGTAGAGGTTTGACATTCGCCTGCCTCG 3000

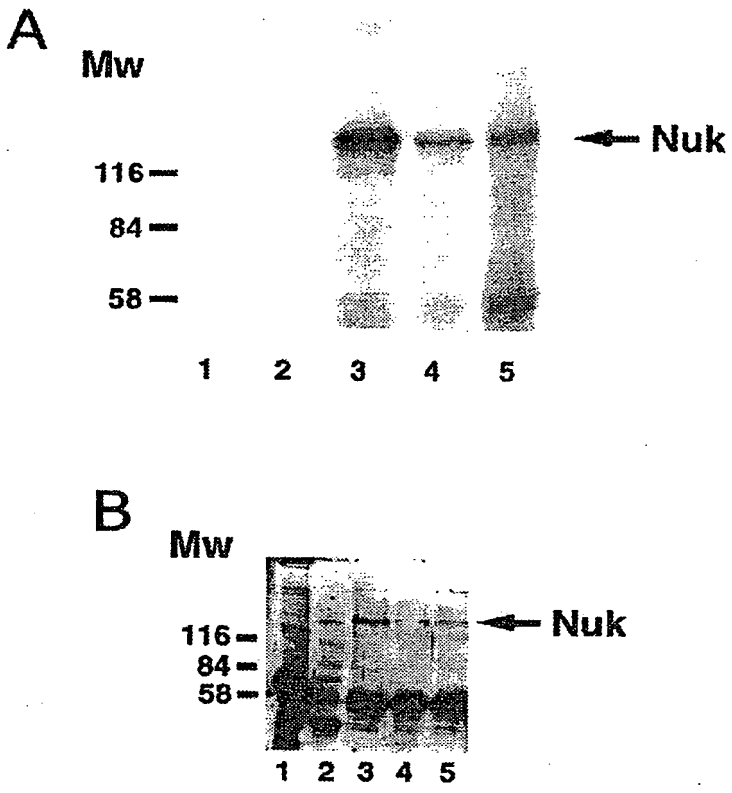
GTTCTCCTCTTCCTCCACGCCGCCCCTGAGCCCCTACGTCGGTCCCTGCTGCTCTGTCAC 3060

TGCAGGTCAGCACTGCCAGGAGGCCACAGACAACAGGAAGACCAA 3105

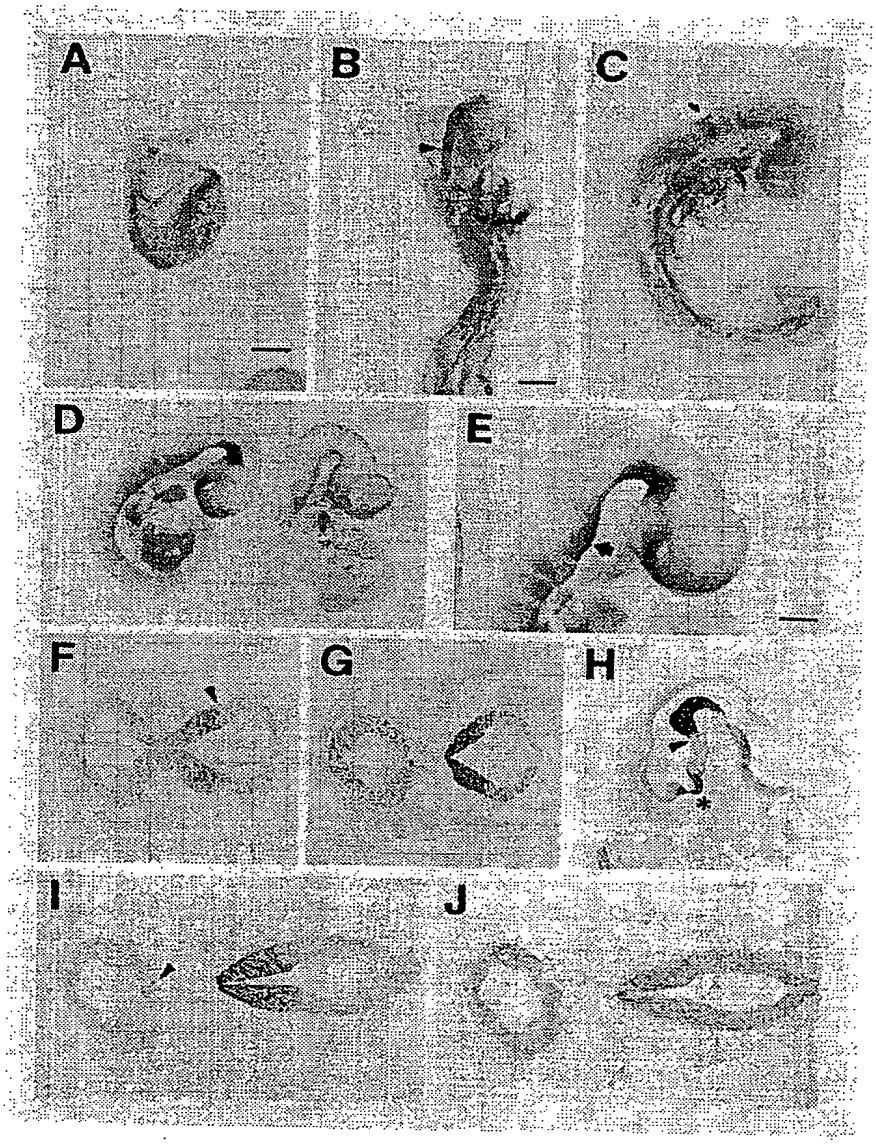
FIGURE 2



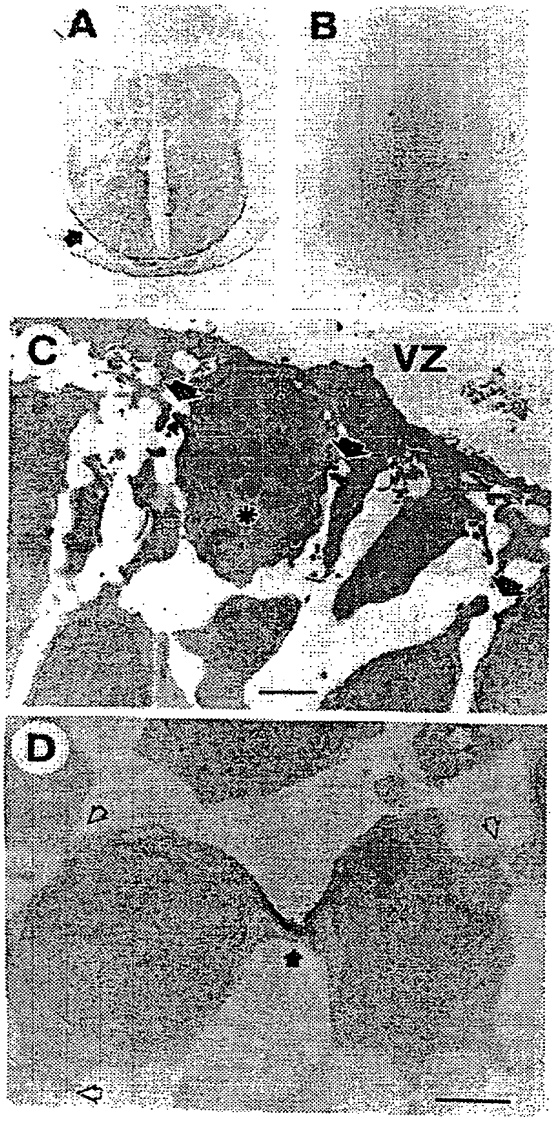
FIGURES 3 A-B



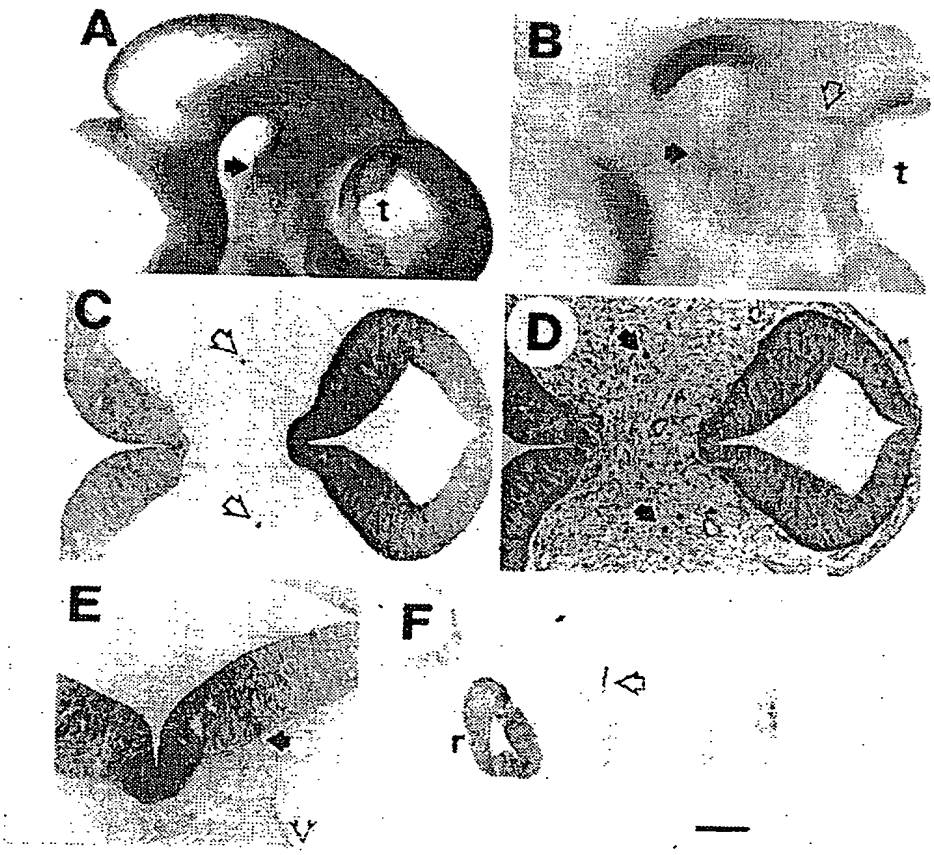
FIGURES 4 A-J



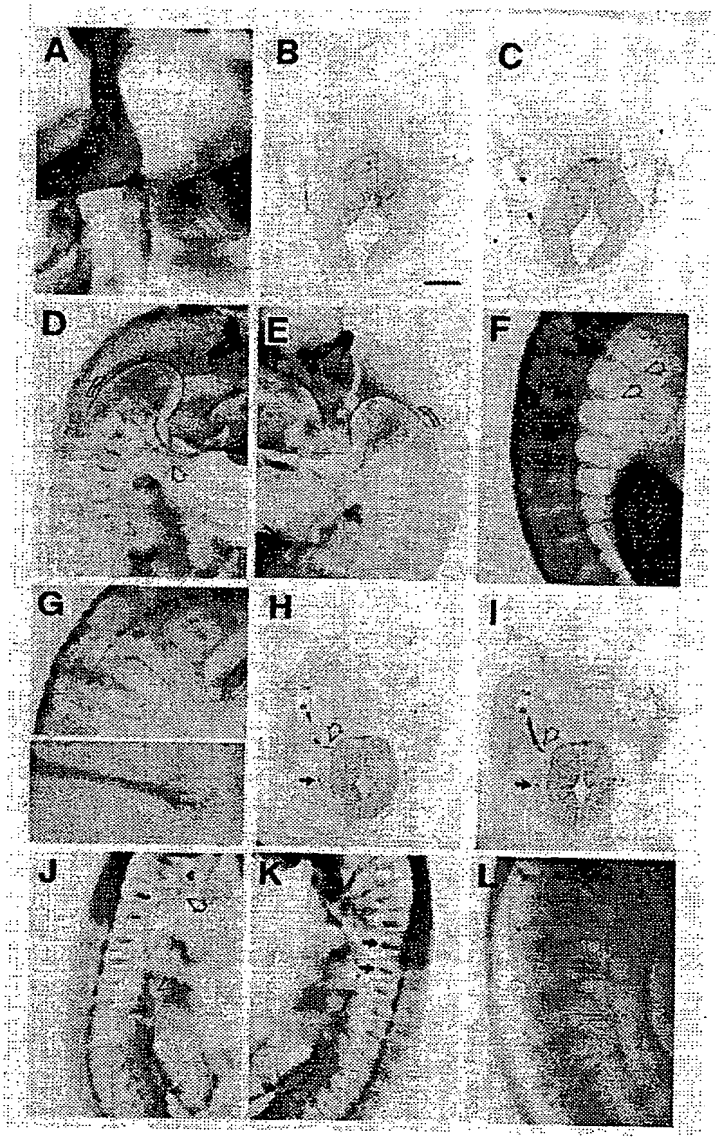
FIGURES 5 A-D



FIGURES 6 A-F



FIGURES 7 A-L



FIGURES 8 A-D

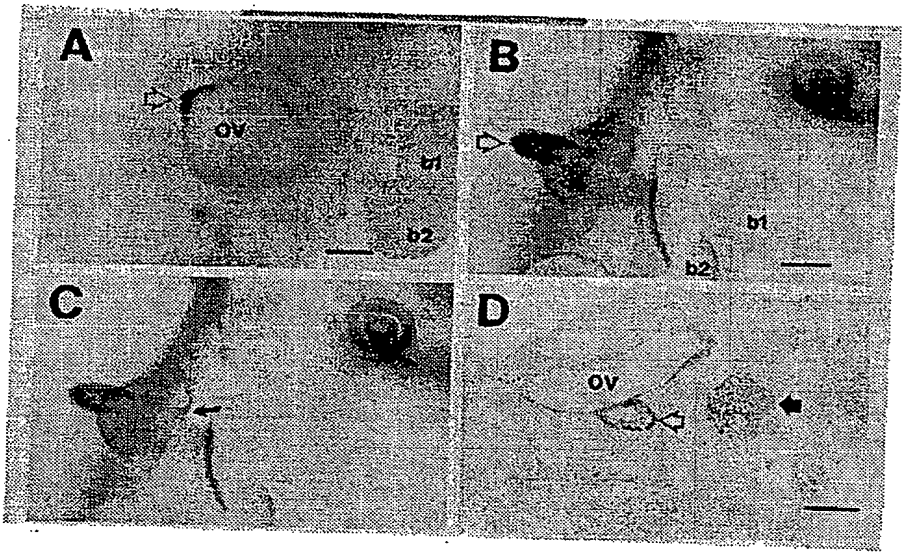
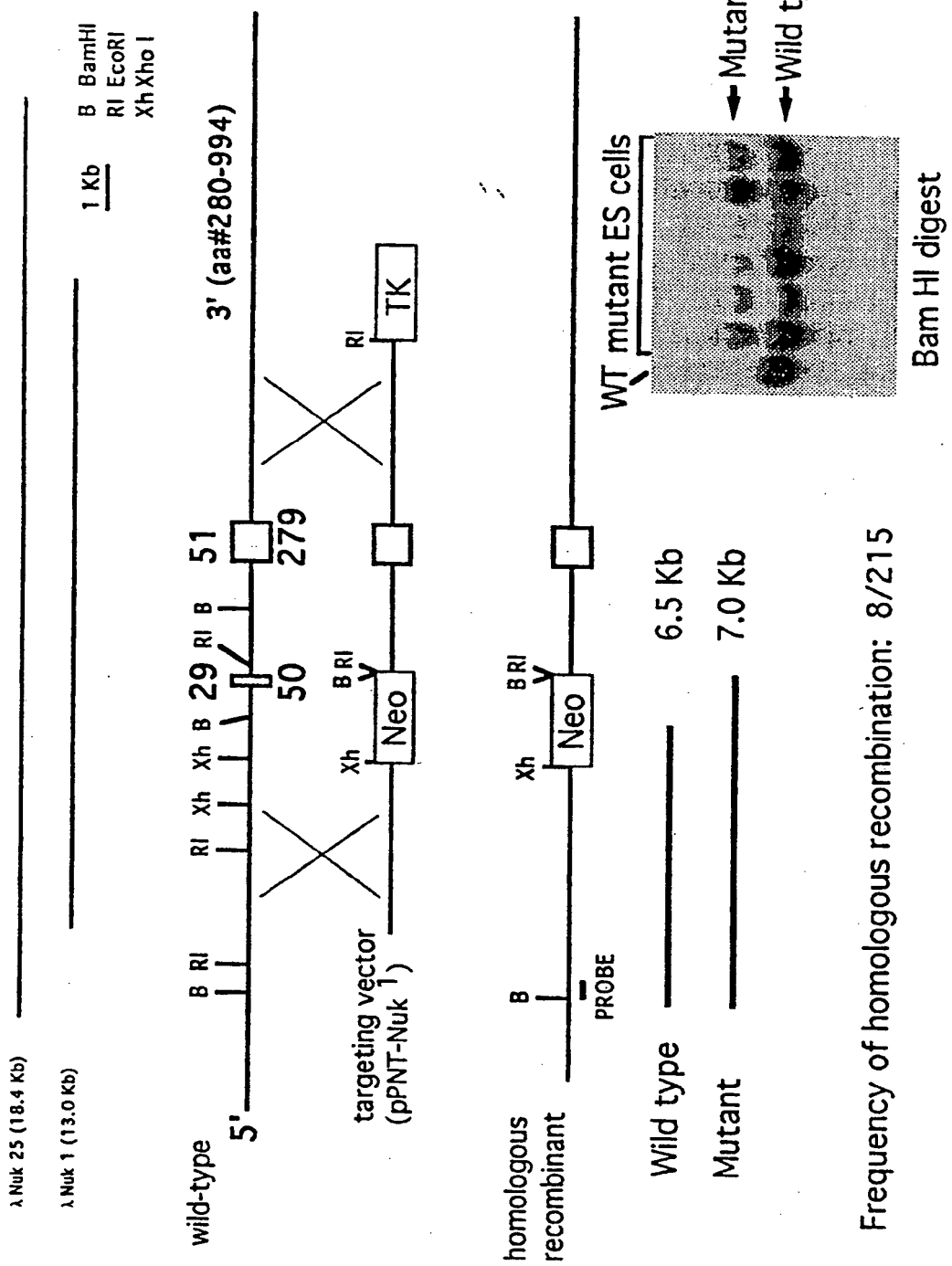


FIGURE 9

Nuk gene targeting: deletion of exon 2 (codons 29 to 50).



Frequency of homologous recombination: 8/215

FIGURE 10

The *Nuk* ^{2} mutation expresses a Nuk- βgal fusion protein lacking the tyrosine kinase catalytic domain.

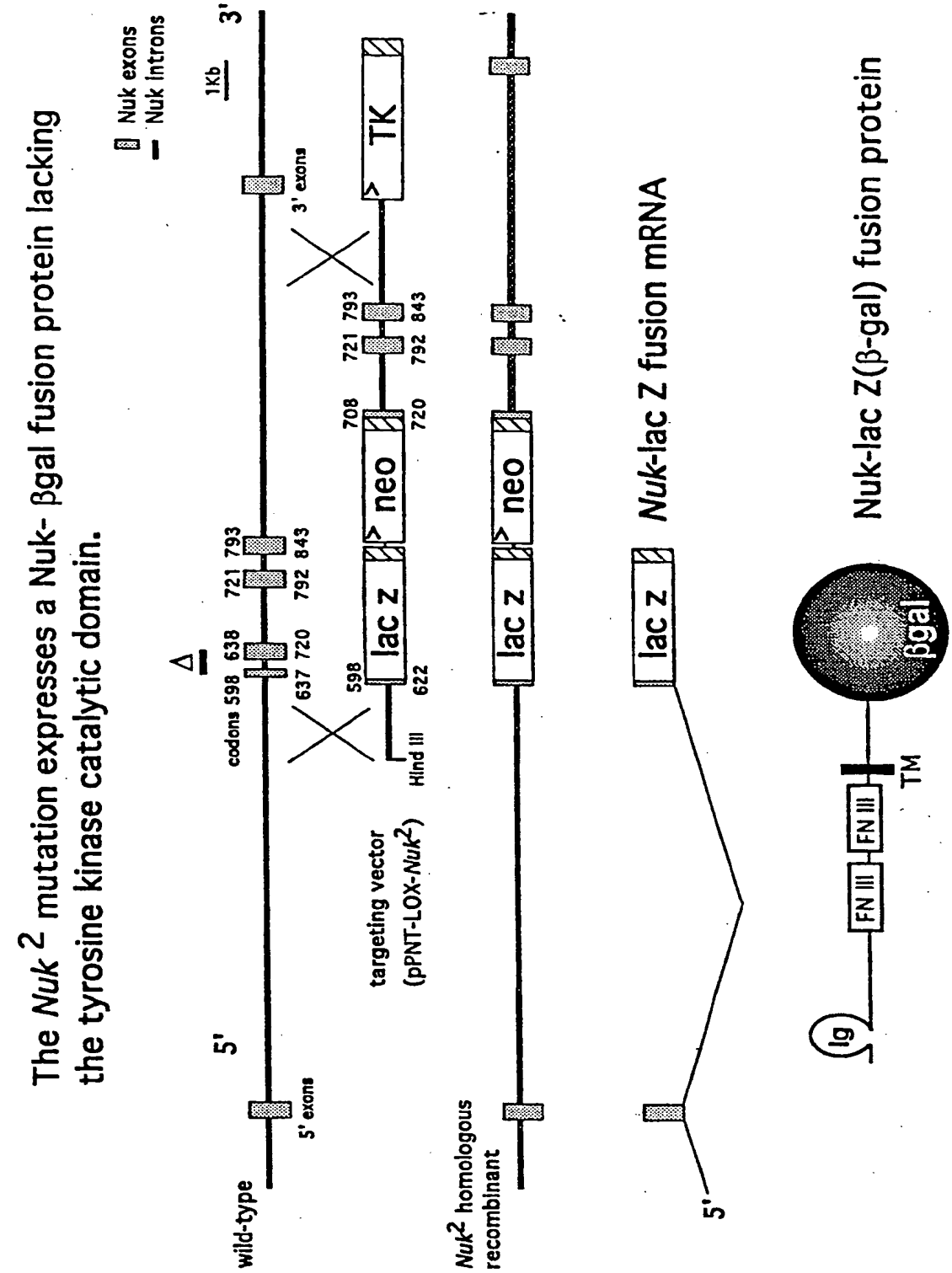


FIGURE 11 A

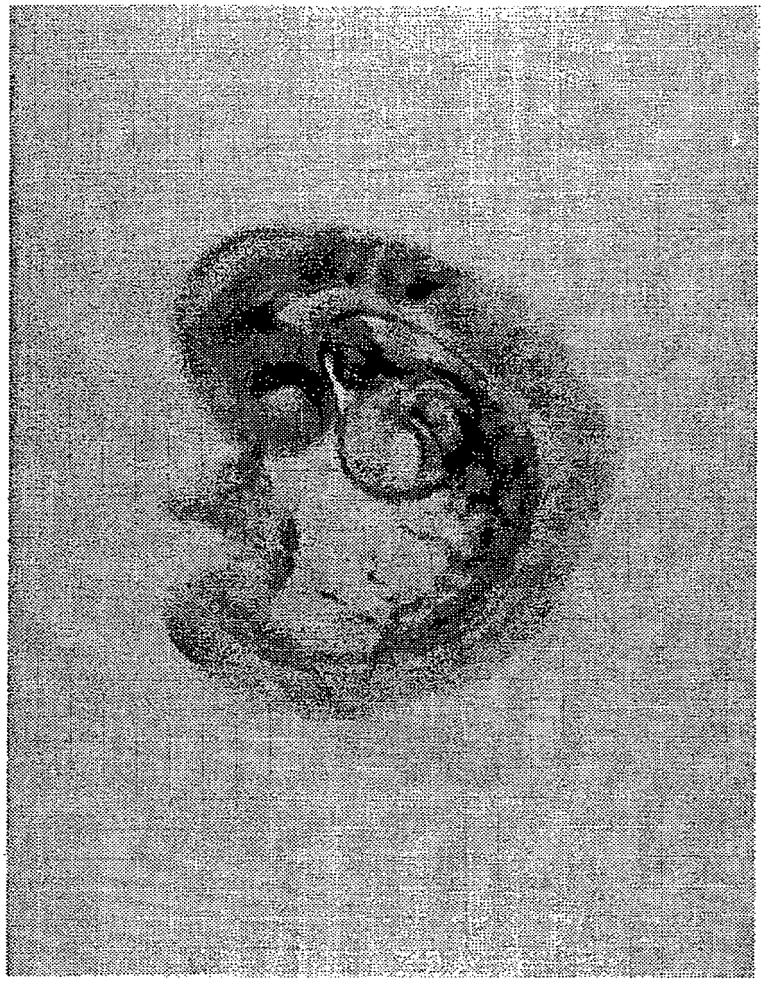


FIGURE 11 B

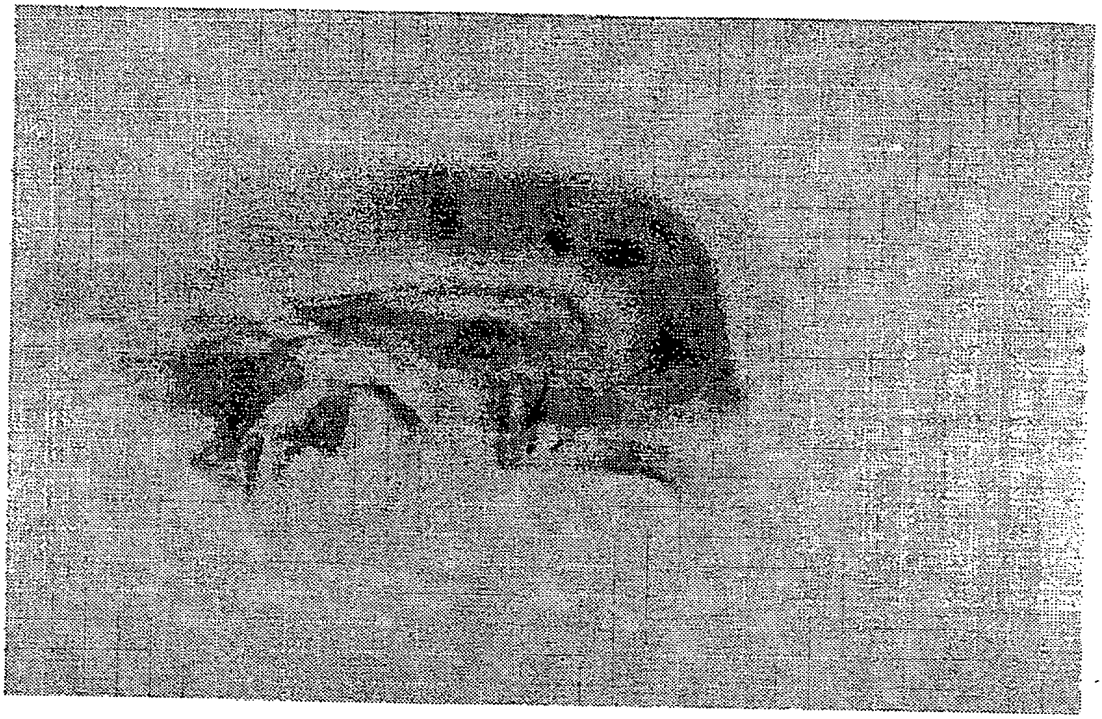


FIGURE 12 A

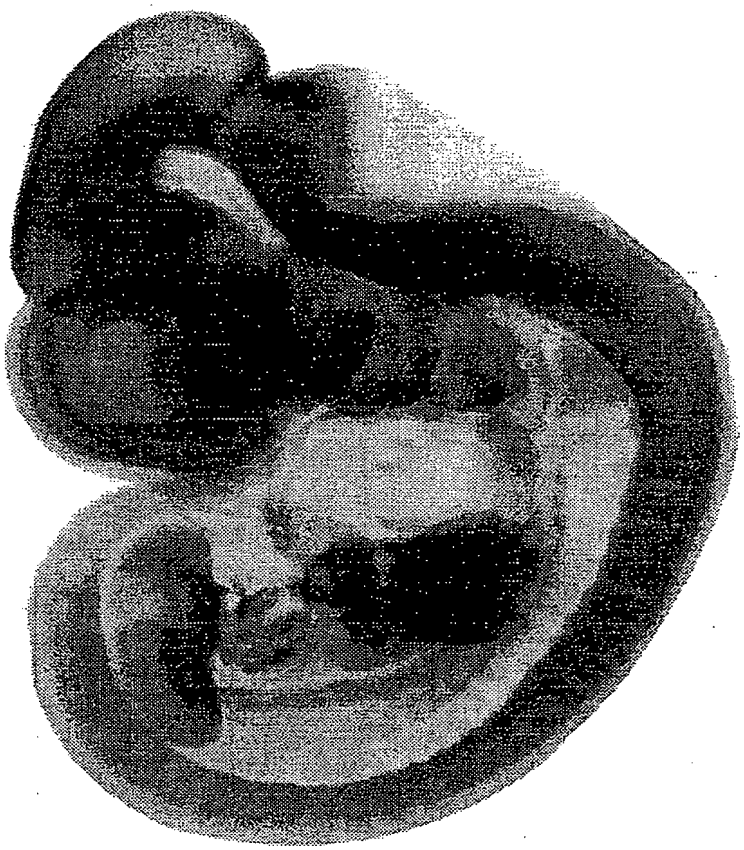


FIGURE 12 B

FIGURE 13

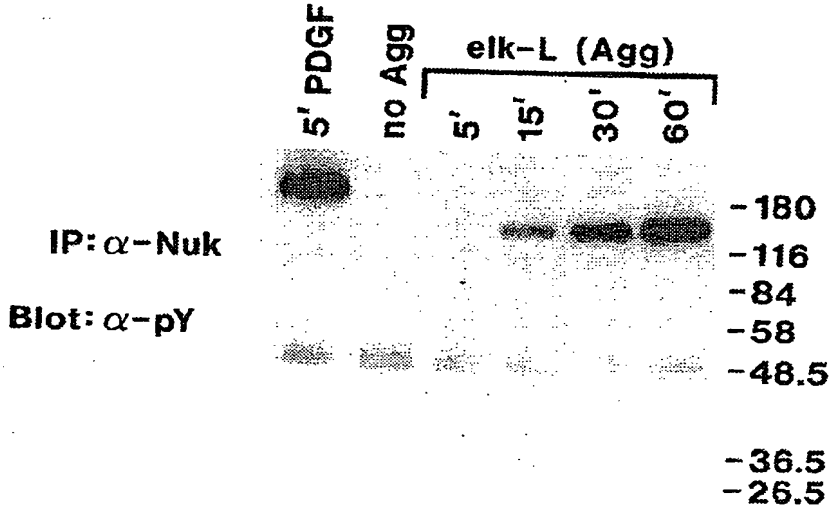


FIGURE 14

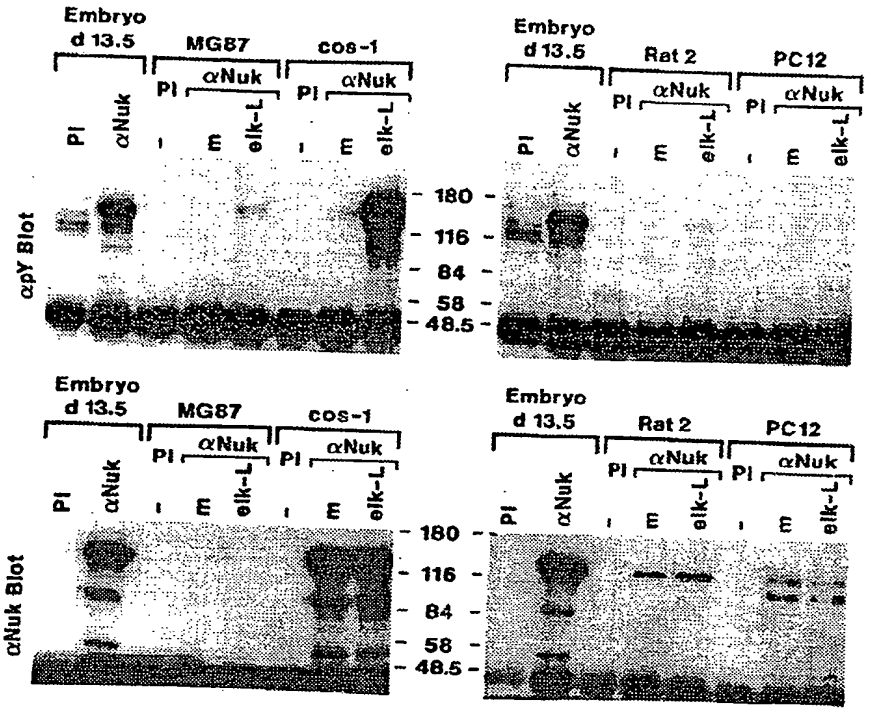


FIGURE 15

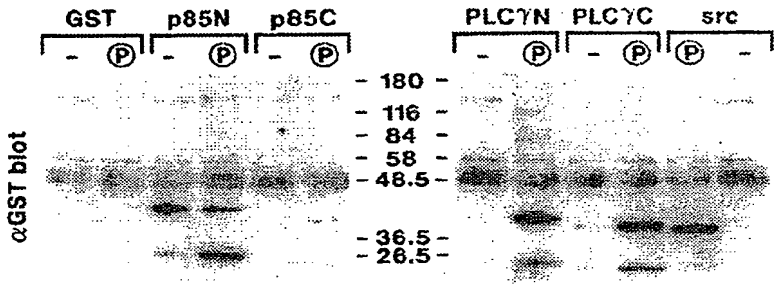


FIGURE 16

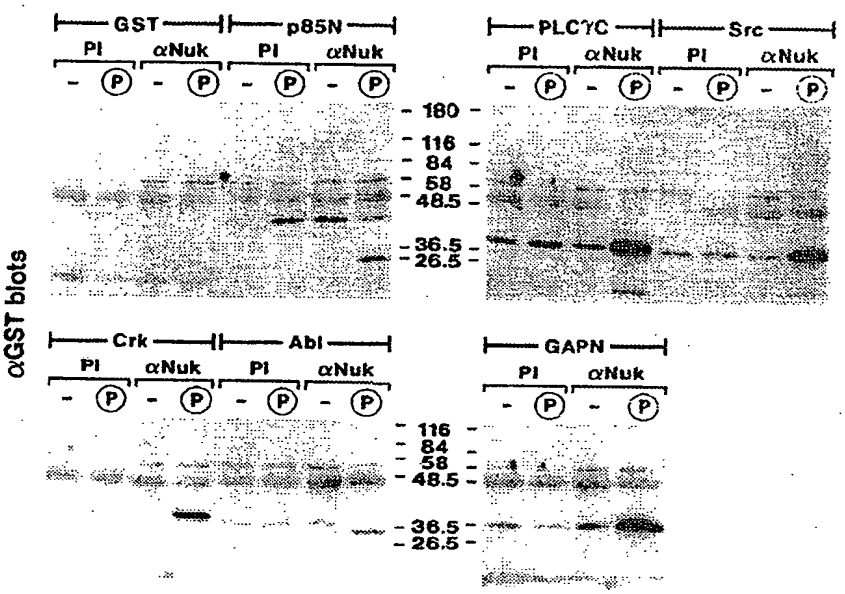


FIGURE 17

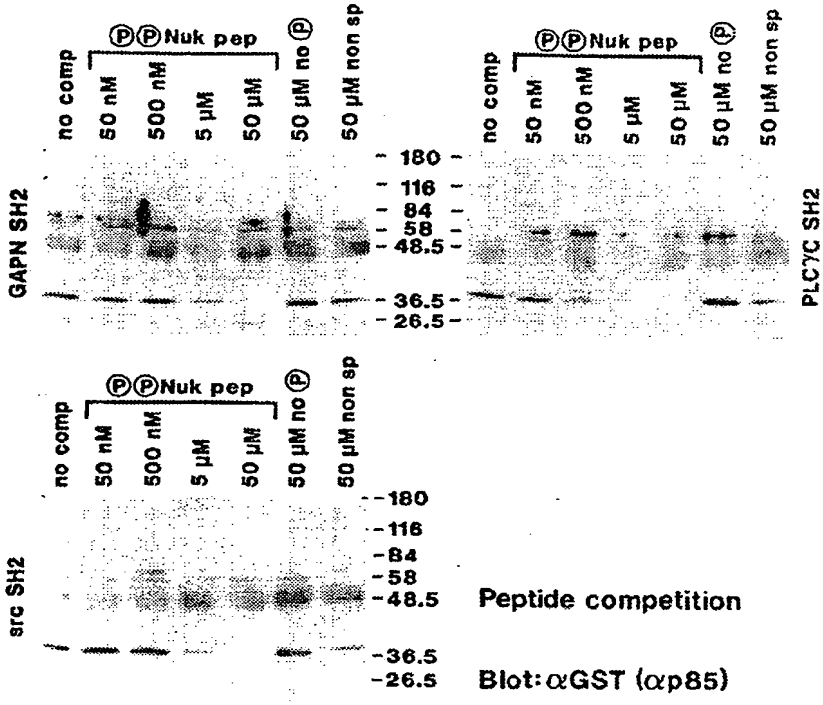
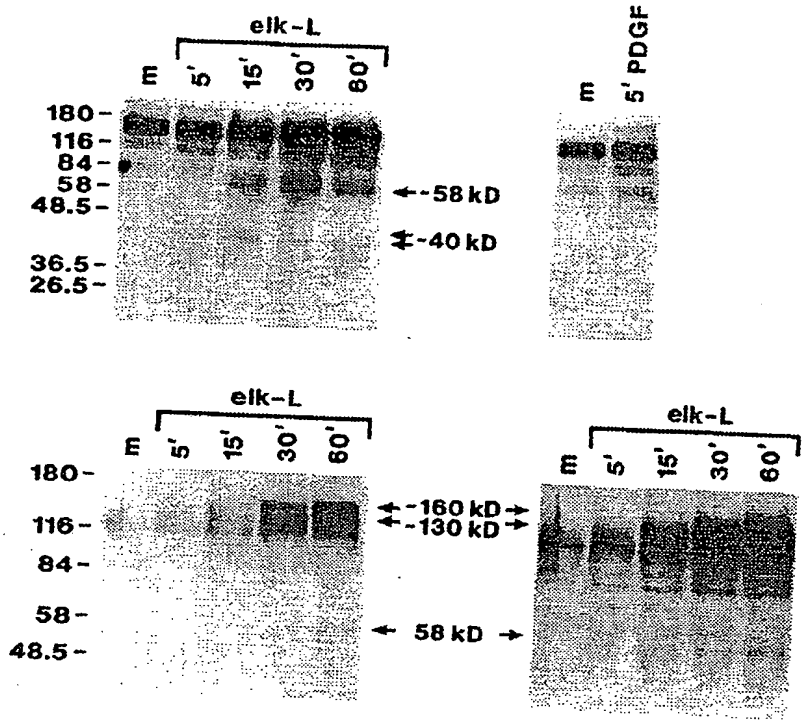


FIGURE 18



cos-1 lysate
Blot: αpY

INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/CA 95/00254

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N9/12 C12N5/10 C12Q1/48 C07K16/40
C12N15/00 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL RODENTS DATABASE, Accession no. L25890, sequence reference MMNRTK, November 6, 1993; M. HENKEMEYER: "Immunolocalization of the Nuk receptor tyrosine kinase suggests a role in segmental patterning of the brain and axonogenesis" see the whole document ---	1-8,14, 15
X	ONCOGENE (1994), 9(4), 1001-14 CODEN: ONCNES;ISSN: 0950-9232, April 1994 HENKEMEYER, MARK ET AL 'Immunolocalization of the Nuk receptor tyrosine kinase suggests roles in segmental patterning of the brain and axonogenesis' see the whole document --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 1995	13. 10. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

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INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/CA 95/00254

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KEYSTONE SYMPOSIUM ON GROWTH AND DIFFERENTIATION FACTORS IN VERTEBRATE DEVELOPMENT, KEYSTONE, COLORADO, USA, APRIL 3-10, 1992. J CELL BIOCHEM SUPPL 0 (16 PART F). 1992. 106. CODEN: JCBSD7, HENKEMEYER M ET AL 'THE MURINE RECEPTOR TYROSINE KINASE NUK IS CONCENTRATED IN A SUBSET OF CELL-CELL JUNCTIONS DURING EMBRYOGENESIS.' see the whole document	1-16
A	WO-A-93 00425 (INST MEDICAL W & E HALL) 7 January 1993 see page 9, paragraph 3 - page 10, paragraph 1	9-12
A	CELL, vol. 75, 17 December 1993 pages 1157-1167, S.LYMAN ET AL 'Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor' cited in the application see page 1157, right column, paragraph 3 - paragraph 3	9-12
A	EMBO JOURNAL, vol. 9, no. 10, 1990 pages 3279-3286, A. KAZLAUSKAS AND J. COOPER 'Phosphorylation of the PDGF receptor beta subunit creates a tight binding site for phosphatidylinositol 3 kinase' see the whole document	13

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internation Application No
PCT/CA 95/00254

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9300425	07-01-93	AU-B-	655299	15-12-94
		EP-A-	0590030	06-04-94
		JP-T-	6508747	06-10-94

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